TEXT-BOOK OF BACTERIOLOGY

By

R.W. FAIRBROTHER.

Fifth Edition. 1948.

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Bacteriophages.
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A TEXT-BOOK OF BACTERIOLOGY

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BY

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PREFACE TO THE FIFTH EDITION

RELEASE from Service activities and the return to the amenities and relative tranquillity of a civilian laboratory have made possible a thorough revision of the text. Obsolete material has been removed and an attempt made to incorporate, in a form suitable for the student, the many important developments in bacteriology that have taken place during recent years.

It is a pleasure to thank Prof. A. W. Downie for much valuable criticism, Dr. S. T. Cowan for advice on, and the illustration for, the section on penicillin, and my wife for assistance with the index.

MANCHESTER, *August*, 1946. R. W. F.

PREFACE TO THE FIRST EDITION

THIS book is an outline of the medical aspects of bacteriology. The field of bacteriology has been so greatly extended during the past 10 or 15 years that no account of the whole of it, even in outline, is possible in the short time available in the medical curriculum. The medical student must therefore restrict his attention to those branches of the subject that are of medical importance, viz., bacteria as agents of disease in man, and the application of bacteriological methods in the prevention, diagnosis and treatment of disease. Few of the available text-books stress these points adequately, and the presentation of principles is often interrupted by masses of technical detail needed only in the laboratory.

In the present book an attempt is made to meet these criticisms. In the absence of a universally accepted bacterial nomenclature, that recommended by the Society of American Bacteriologists is followed, and the biological relationship of allied organisms is emphasized.

The bacteriological works consulted during the writing of this book are too numerous to list in full, but my great indebtedness to the "Principles of Bacteriology," by W. W. C. Topley and G. S. Wilson, and to the "System of Bacteriology" published by the Medical Research Council, demands specific acknowledgment.

I wish to thank Professor H. B. Maitland, Dr. D. T. Robinson and Dr. R. Whitehead for advice and criticism, Dr. W. Susman and Mr. V. H. Moore for the various photographs, and my wife for the drawings, the coloured plates and much general assistance.

R. W. F

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A TEXT-BOOK OF BACTERIOLOGY

PART I

GENERAL BACTERIOLOGY

CHAPTER I

HISTORICAL SURVEY

ALTHOUGH there is no doubt that bacteria were observed almost three centuries ago, their significance in the production of disease was not established until the brilliant work of Louis Pasteur, a French chemist, some eighty years ago. Previous to this period much had been written about the nature of disease and the spontaneous generation of living things, but these views were largely the speculations of philosophers and in only a few cases were they subsequently proved to be correct. These speculations, however, were mainly responsible for the development of bacteriology, and a brief review of their salient features is both interesting and instructive.

It has been long recognized that disease might be communicated from one individual to another; there are numerous references in the works of the earlier writers to indicate this, particularly in connection with plague and small-pox, which frequently appeared in epidemic form during the Middle Ages. The nature of the infecting material was not, however, determined; Fracastor of Verona (1546) put forward the view that disease was spread by particles too small to be seen and termed the responsible agent a *contagium vivum*. He divided the contagious diseases into two groups: those transmitted *per contactum*, *i.e.*, from person to person, and those transmitted *per fomitem*, *i.e.*, through the agency of some outside object. The first person to suggest, on experimental grounds, that disease might be caused by visible living bodies was a monk, Kircher. Kircher, in 1671, using simple lenses of low magnification, stated that he had observed peculiar "worms" in the blood of persons suffering from plague. Although he considered that these were the agents responsible for the disease, there seems to be little doubt that the objects he saw were simply arrangements of the red blood corpuscles.

A short time afterwards the existence of minute living bodies was definitely established. Leeuwenhoek, a Dutch draper whose hobby was the preparation of lenses, introduced the simple microscope during the last quarter of the seventeenth century. Using these instruments, with which magnifications of from 40 to 300 diameters were obtained, he observed minute bodies which he termed "animalcules". These were present in considerable numbers in rain water and scrapings collected from the spaces between the teeth. Some of the bodies were shaped like bent sticks and others like spirals, while many were actively motile. Leeuwenhoek described his results in numerous letters to the Royal Society of London, but he did not appreciate that the animalcules might have some connection with the production of disease. There appears to be no doubt that Leeuwenhoek actually saw the larger bacteria, which are normally present in water and the mouth. An Austrian, Plenciz, in 1762 postulated the germ theory and the specificity of disease. Plenciz, as a result of experimental work of doubtful value, concluded that each disease had its own specific agent which multiplied in the body and so produced disease.

At this time, the middle of the eighteenth century, a considerable amount of controversy was taking place on the question of spontaneous generation. After Leeuwenhoek had described the peculiar minute bodies, or animalcules, their presence in various situations was confirmed without difficulty. The adherents of the theory of spontaneous generation considered that by an investigation of the animalcules proof of this theory could be definitely established. Needham, about 1750, communicated to the Royal Society the results of his experiments on the generation of these minute bodies. He heated meat-broth at temperatures, as he thought, sufficient to destroy living things and left it tightly sealed for some days. On subsequent examination Needham found that the broth was swarming with the animalcules. By that and similar experiments he considered that the spontaneous origin of the animalcules was proved.

These views, however, did not satisfy an Italian priest, Spallanzani, who prepared similar flasks of meat-broth and boiled them for varying periods of time. One set was sealed and the other corked; they were then stored for several days. On examination it was found that the animalcules were present in all the flasks except those which had been sealed after being boiled for some time; they were present in those flasks that had only been boiled for a short time. Spallanzani considered that the minute bodies could resist boiling for a short time and that spontaneous generation had not taken place; the living bodies had simply developed from the living bodies already present in the medium. Needham in reply to these criticisms stated that the experiments of Spallanzani were valueless, as by the prolonged heating of the meat-broth some hypothetical substance, which he termed "vegetative force", had been destroyed. This vegetative force he considered was necessary for the production and development of the animalcules. This objection was overcome by Spallanzani; he found that the animalcules would grow readily in meat-broth that had been subjected to high temperatures for prolonged periods of time. As a result of further experiments Spallanzani was able satisfactorily to counter the hypotheses advanced by the adherents of the doctrine of spontaneous generation. This theory subsequently became more and more discredited, although some naturalists continued to uphold it for many years.

The development of the microscope was also advancing rapidly, and higher magnifications were soon available for the study of minute bodies. It must be realized that, at this stage, the only means of examination was by the observation through these early microscopes of unstained material. In spite of these difficult technical conditions definite progress was made; Ehrenberg, 1838, described various forms and classified four groups: *vibrio*, *bacterium*, *spirillum* and *spirochæta*. A short time previously, 1837, Latour and Schwann had found yeasts in beer and wine and considered that they were responsible for the fermentation process.

Although some of these early observations were undoubtedly accurate, many conflicting statements were also made on most unsatisfactory experimental findings. This haphazard manner of investigation was criticized appropriately by Henle, a famous anatomist, in 1840. Henle emphasized that, before ætiological relationship of a micro-organism to a disease process could be established it must be found constantly in cases of the disease and it must be isolated and tested by inoculation into healthy animals. These arguments were amplified later by Koch in his now-famous postulates.

At this time the question of spontaneous generation again came to the fore. The observations of Schwann and Latour, that yeasts were responsible for the fermentation process in beer and wine, were subjected to much criticism. Liebig, a celebrated German chemist, stated that fermentation was purely a chemical process and that the ferment was obtained from the dead protein already present in the reacting system. In view of his reputation, Liebig's views were almost generally accepted; this did much to retard progress.

It was at this stage, 1856, that Pasteur entered the realms of bacteriology. Originally a chemist, Pasteur undertook an investigation into the cause of some faulty fermentation which was seriously affecting the wine industry of Lille. As a result of his work, Liebig's theory of fermentation was finally and completely disproved; Pasteur found that yeasts were responsible for the fermentation process and that sugar could be converted into alcohol in the absence of any protein in the medium. He also showed that faulty fermentation was the result of the presence of some contaminating rod-shaped micro-organisms. The theory of spontaneous generation was also finally crushed by further researches of Pasteur, in which innumerable flasks with wide, constricted, twisted and elongated necks were made in order to show that the contaminating micro-organisms had their origin in the air. The effect of heat on these bodies was also examined, and he found that certain organisms were able to withstand boiling for some time; these were doubtless the spore-bearing bacteria. Some organisms were found to grow in the absence of oxygen while others required its presence. The use of cotton-wool plugs in order to prevent organisms from entering flasks already sterilized was also demonstrated about this time by Schroeder and Dusch. The relationship of micro-organisms to the processes of fermentation and putrefaction thus became definitely established, but it is interesting to note that up to this time conclusive evidence that these minute bodies were agents of disease had not been obtained.

After his brilliant work on the question of fermentation, Pasteur (1865) was next persuaded to investigate a disease of silkworms, pébrine, which was crippling the silk industry of France. Although mishaps and setbacks were frequent, Pasteur, after five years' work, had settled the problem and re-established the silk industry on a prosperous basis. He found that the disease of the worms was due to some living microscopical organism and that the disease was transmitted from one animal to another. By obtaining healthy silkworms and subsequently removing any showing signs of infection Pasteur was able to maintain a healthy stock, although he was surrounded by infected farms. The relationship of living microscopical objects to the disease was thus proved ; it is a point of interest that this disease was of protozoal and not bacterial origin.

These important fundamental observations of Pasteur were not allowed to pass unnoticed by the outside world. Although there were many who decried these new and revolutionary ideas, there were others who realized the true significance of this work. Lister, then Professor of Surgery at Glasgow University, considered that the micro-organisms, so prevalent in the air, might be responsible for the frequent occurrence of the inflammation and suppuration after surgical operations. In spite of much criticism and opposition, he, in 1867, revolutionized surgery by introducing an antiseptic technique. In this method sprays or washings of carbolic acid were directed during the operation on the wound, which was subsequently protected from the air by suitable dressings. The results which followed this procedure were remarkably successful and the practice became increasingly popular ; there followed a striking reduction in the incidence of post-operative sepsis. This was an important demonstration of the *rôle* played by micro-organisms in the causation of disease. A later advance was the replacement of antiseptic surgery by aseptic surgery.

The study of the micro-organisms, although a matter of some difficulty, was now becoming increasingly popular. Many varied forms were seen and described. A further and more systematic method of classification was elaborated by Cohn (1872) ; in this were included the following generic names—*micrococcus*, *bacterium*, *bacillus*, *vibrio* and *spirochæta*. The term *bacterium* was also used for the first time to designate the micro-organisms multiplying by fission. This classification was based on morphological observations, and it is interesting to note that many of the terms are used to-day in the sense originally employed by Cohn, who also described the appearance of spores in a non-pathogenic bacillus and noted the increased resistance to heat and disinfectants possessed by the spore-bearers.

About this time the relationship of a micro-organism to an important animal disease, splenic fever or anthrax, was being investigated. Pollender, 1849, and Davaine, 1850, had described the presence of a rod-shaped organism in the blood of animals suffering from splenic fever. Some time later Davaine reproduced the disease in healthy animals by injecting them with blood collected from infected animals. The connection between this organism and the disease, however, was not definitely established until the thorough and brilliant work of a medical practitioner in Germany, Robert Koch. Koch examined the organisms present in the blood of infected animals, reproduced the disease in healthy mice by inoculating them with infected blood and also prepared cultures by inoculating, on a hollow ground slide, the aqueous humour of a bullock's eye with the infected blood. The growth of the organisms in culture was observed, and after several subcultures the organism was still found to be virulent for the mouse. The production of spores by the rods and the vegetation of the spores into rods were also observed and described. In this manner, in 1876, the ætiology of anthrax was established beyond any shadow of doubt. This observation was confirmed and elaborated shortly afterwards by Pasteur, who introduced a method of active immunization whereby the resistance of animals to the disease was increased. Pasteur had previously found by accident that the inoculation of chickens with relatively avirulent cultures of the organism of chicken cholera did not produce the disease and that a later inoculation of these birds with a virulent strain was also unsuccessful. This result indicated to Pasteur that the original avirulent culture had protected the chickens against subsequent infection with the virulent organisms. This was a step of great fundamental importance; although prophylactic immunization against small-pox by means of active cow-pox material had been developed and practised by Jenner in 1800, this procedure was entirely empirical.

Pasteur next endeavoured to apply his discovery to splenic fever. Here, owing to the presence of the resistant virulent spores, the difficulty was to obtain a relatively avirulent culture of the anthrax bacillus. This, however, was overcome by growing the organisms at a temperature higher than that optimal for growth and at which spores did not develop. This attenuated culture was given and then followed after a short interval by a second inoculation of a less attenuated culture. The value of these injections in protecting sheep was demonstrated publicly by Pasteur at Pouilly-le-Fort in 1881; the test was a striking success, the control animals died of anthrax or splenic fever, whereas the protected sheep were quite unaffected by the inoculation of the virulent test-culture. Although further trials were less satisfactory, these observations began the study of protective inoculation against infectious diseases.

Koch meanwhile had become a full-time bacteriologist and was making further important advances in bacteriological technique. The methods employed in the study of bacteria at this time were very primitive ; microscopical observations were made in unstained material, while cultures had been only prepared in fluid media. Many would consider that, under these conditions, the study of bacteria was impossible ; the wonderful results obtained by Koch and Pasteur consequently give some indication of the brilliance of these workers. Koch soon made several important improvements ; in 1877 he prepared dried films which he stained with certain of the aniline dyes ; he also improved the microscope by using an Abbé condenser and oil-immersion lenses, which had been employed in spheres other than bacteriology. Shortly afterwards he introduced another step of great value, viz., the use of solid media. The restriction to the use of fluid media for the cultivation of bacteria rendered the preparation of pure cultures a matter of considerable difficulty. The introduction by Koch of gelatin as a solid media was an important advance in bacteriological technique, as growth was now obtainable in the form of colonies, which could be readily selected and picked off for further study. A simple means of separating individual organisms from contaminated material was also available. The use of gelatin was followed by the application of serum and later of agar-agar in the preparation of a solid medium. Potato and bread had been employed previously, but the results obtained were uncertain and unreliable. A further improvement was the introduction of satisfactory containers for solid media by Petri ; " Petri " dishes are still employed extensively in the study of bacteria.

Koch in 1882 published his classical investigations on tuberculosis, in which he proved that the tubercle bacillus was the causal agent of the disease. In his reports of this work Koch formulated his famous postulates on the association of a bacterium to its particular disease, viz., a bacterium should always be associated with its particular disease, must be isolated in pure culture and should reproduce the characteristic disease in susceptible animals. While the fulfilment of all these criteria is not considered essential at the present time for establishing the relationship of an organism to a particular disease, their value in checking preposterous claims by inexperienced workers has been considerable.

Bacteriology was now definitely acclaimed, innumerable investigations were carried out and the causal relationship of particular organisms to many important diseases was determined during the following 10–20 years. The significance of bacteria in agriculture and industries, such as dairy-farming, canning, wine-making, etc., also became more and more appreciated.

An advance of fundamental importance was the discovery by Roux and Yersin in 1888 that the symptoms of diphtheria were due mainly to the action of the soluble toxin formed by the diphtheria bacilli. Roux, 1894, subsequently prepared an antitoxin in the horse, the value of which in the therapy of diphtheria cannot be over-estimated. The importance of toxins in certain other conditions, such as tetanus and other anaerobic infections, was later demonstrated.

The observations that infectious diseases of animals might be caused by ultramicroscopic bodies, the filterable viruses, was first made by Loeffler and Frosch in 1898. They found that the causal agent of foot and mouth disease could not be seen and readily traversed the ordinary bacterial filters, and that the disease could be reproduced indefinitely in susceptible animals by the injection of filtrates of the specific lesions. A new field of research was opened and many diseases, both animal and human, were subsequently found to be caused by these disease-agents.

The *rôle* played by bacteria in the causation of disease has thus only been investigated by workers during the past 70 years. The ætiology of a considerable number of diseases has been solved, but in some cases, such as the common cold, the position still remains uncertain. While recent work has proved conclusively that epidemic influenza is primarily a virus-disease, and that at least two antigenically distinct types of the virus exist, the ætiology of sporadic cases of the disease is still unsolved.

It has been found that only a small percentage of the bacteria are pathogenic to man or animals under ordinary conditions. The exclusion of the non-pathogenic members from a disease process is, however, often a matter of considerable difficulty, and this factor has been responsible for many erroneous observations. Much of the work of the last decade has consequently been carried out either to consolidate or to discredit the claims made by earlier workers. Bacteriological technique is gradually improving, and it is quite probable that some of the views accepted at the present moment may be discredited in later years.

It is interesting to note that, until recent years, the major portion of the work carried out on the bacteria was of an applied nature. From a medical standpoint attention had been largely directed to the relationship of an organism to the disease process and to the value of specific prophylactic and therapeutic measures in overcoming infection. Bacterial metabolism and the mechanism of the various immunological reactions have recently been receiving more attention, as it is realized that bacteria are important not only in the production of disease but also in many diverse economic problems.

Assistance from both physicists and chemists has been necessary for the investigation of these complex problems and, as a result, striking advances have been made. It has been found, for example, that the sulphonamides interfere with the normal metabolism of sensitive bacteria and thereby prevent multiplication. Further developments in this field of research will be awaited with interest. Other interesting contributions have been the new methods for the fractionation of plasma and the production of gamma globulins by workers in America.

War-time conditions produce opportunities for the study of problems which are not normally encountered. During the Great War of 1914–18 much progress was made in many branches of bacteriology, striking examples being the ætiology of gas-gangrene and the classification of the anaerobes and the study of cerebrospinal meningitis.

Considerable progress was also made during the recent World War. Chemotherapy developed rapidly. The range of the sulphonamides increased and their activity in such conditions as cerebrospinal meningitis, bacillary dysentery, gonorrhœa and war wounds was investigated in great detail. Penicillin was introduced as a therapeutic agent under strict official control and, in consequence, its value and also its limitations are now well appreciated. Furthermore, the great need for the prevention of disease and conservation of man-power led to careful studies and important advances in specific prophylaxis against tetanus, typhus fever, yellow fever, enteric fever and small-pox. Much progress was also made in the study of cross-infection, particularly in hospital wards. A considerable amount of work was carried out on infective hepatitis and atypical pneumonia and valuable contributions to our knowledge of these subjects were made, but in view of the lack of suitable experimental animals, progress was slow and much still awaits solution.

CHAPTER II

THE MORPHOLOGY OF BACTERIA

BACTERIA are extremely minute, chlorophyll-free, unicellular bodies, which multiply by simple transverse division or fission. They have also been designated at various times by such names as "germs, microbes, micro-organisms". Of these, the term "bacterium" has been the one most widely applied, and it is perhaps unfortunate that it has also been adopted as a generic name for a particular group of rod-shaped forms.

The bacteria are placed at the lower limit of the scale of living bodies and they have affinities with the lower forms of both plant-life, *e.g.*, the algæ, moulds and fungi, and animal life, *e.g.*, the protozoa. The following are the main points of difference between these groups and the bacteria : the protoplasm of the algæ contains chlorophyll ; the fungi and moulds are multicellular and possess a sexual phase of reproduction ; the yeasts reproduce by budding ; the protozoa have a definite nucleus and a sexual phase of reproduction. Bacteria are generally accepted as a special class of the plant kingdom, which has been designated *Schizomycetes*. This is subdivided into orders, families, genera and species.

The individual bacterial species may exhibit wide variations in size and shape, but when grown under favourable conditions they tend to present features that are reasonably constant. Under such circumstances valuable information of the identity of an organism can be obtained by a study of its form and structure. This is carried out by an examination of either unstained or stained preparations.

Examination of Unstained Material. This may be undertaken in routine examinations, with living or dead bacteria either by direct microscopy or by the use of dark-ground illumination ; in both cases a suspension of the bacteria is necessary. In carrying out the examination by direct microscopy use is made of the hollow-ground slide, which is a slide with a hollow of approximately ½ in. ground out on one surface (Fig. 1). The surface around the hollow is covered with a thin layer of vaseline ; a drop of the bacterial suspension is placed on the centre of a clean cover-glass, the slide is then gently lowered on to the cover-glass so that the drop lies undisturbed in the hollow. The slide is then turned over and the drop examined by the high-power objective ($\frac{1}{6}$ in. lens) after its edge has been located with the low-power lens. The vaseline fixes the cover-glass and also tends to prevent any evaporation during prolonged observation. The examination should be performed with a diminished intensity of light ; this can be obtained by partial closure of the diaphragm and lowering the condenser or by removing the condenser and using the concave mirror. The rays of light are refracted by bacteria and other particulate bodies present in the suspension, and they therefore appear as dark objects.

The use of dark-ground illumination is reserved for special occasions, such as in the examination of serous exudates for the presence of spirochætes, which are not sufficiently refractile to be visible with the ordinary method of illumination ; it does not usually constitute part of a routine examination. A microscope is required with a special condenser, in which the lenses are arranged so that the direct rays of light are stopped and the material on the slide is illuminated by oblique rays and only those refracted by objects in the field are seen by the observer. Micro-organisms are consequently seen as bright objects against a dark background.

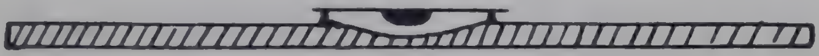


Fig. 1.—A " hollow-ground slide " preparation in section.

The examination of unstained material provides useful information about the bacteria, particularly with regard to their shape, relative number, the mechanism of division, the tendency to any particular arrangement and the presence or absence of motility.

An important development in recent years has been the introduction of the Electron Microscope. By this complex instrument magnifications up to 100,000 times can be obtained and consequently more detailed study of the morphology of bacteria and viruses has been possible. A powerful electron beam passes *in vacuo* through magnetic fields, replacing the usual optical system of the ordinary microscope, and an image of the material under examination can be either seen or photographed. Only a few of these elaborate and expensive microscopes are in existence and they are merely of academic interest to the student.

Examination of Fixed and Stained Material. The observations made on the living material can be extended and elaborated by further examinations of fixed and stained preparations. The film or smear is usually prepared with the platinum loop and is allowed to dry under ordinary atmospheric conditions, after which it is fixed, usually by passing the slide once or twice through the flame of a bunsen burner. Fixation may also be carried out by the addition of such substances as alcohol, mercuric chloride and formalin; these chemical agents must be completely removed by washing in tap-water before the staining-process is commenced. Only the general features of staining are considered here, the detailed technique of the various processes is given later (Chapter XXXVI.).

Bacteria are usually stained readily by the aniline dyes. These are derivatives of the coal-tar product aniline, $C_6H_5NH_2$, and are divided into basic and acid dyes. The basic dyes, monovalent acid salts of dye bases, are the more satisfactory for the staining of bacteria. Those most frequently employed are :—

Basic fuchsin.
Methylene-blue.
Safranin.
Gentian-violet.
Methyl-violet.
Crystal-violet.

These dyes are dissolved in either water or alcohol and are conserved in concentrated solutions, as these are more stable than the weaker solutions. Dilute aqueous solutions, which are made when required, are applied to films for varying periods of time—a few seconds in the case of methyl-violet and carbol-fuchsin to several minutes for methylene-blue. The stain is washed off with water, the film dried between layers of blotting-paper and examined, when dry, under the oil-immersion lens.

The mechanism of the staining of bacteria is not fully understood. It is probable that both physical and chemical factors are involved. The physical process consists in the dye being adsorbed on to the surface of the bacterium, and the chemical process in a chemical combination between the bacterial protoplasm and the dye.

Students should be acquainted with the following terms, which are frequently used in connection with staining :—

A "mordant" is described as a substance which facilitates a particular staining reaction; examples are phenol and weak alkalies, such as caustic potash and ammonium carbonate.

A "decolorizing agent" is a substance which removes the stain; those frequently used in bacteriology are acids, alcohol and methylated spirits.

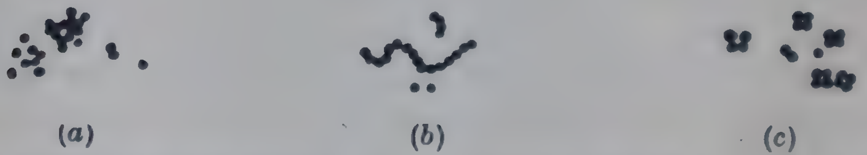
Differential Staining. By the use of the simple aqueous stains all bacteria and much of the extraneous tissue in the film are stained in a more or less uniform manner. Under such conditions the bacteria may be difficult to detect; this is particularly noticeable when necrotic and degenerating tissue is present in the material under examination. The introduction of a differential staining method by Gram in 1884 was consequently a step of great importance. Gram's method is now universally employed, and, although the original technique has been modified by different bacteriologists, the principles involved are essentially the same. Smears are stained, for a short time, with a pararosanilin dye, such as gentian-violet or methyl-violet, treated with an iodine solution (Gram's or Lugol's iodine), decolorized with alcohol, methylated spirits or acetone, washed well with water and counterstained with weak safranin or carbol-fuchsin.

Stained by this method the bacteria fall into two groups: some retain the violet stain and are termed Gram-positive, others, being decolorized, take the counterstain and are called Gram-negative. This simple method of sub-division of the bacteria forms a prominent part of the examination of material containing bacteria. It is important to note that, when correctly stained, a Gram-negative micro-organism is always Gram-negative; a Gram-positive organism, however, may, at certain stages of growth, appear to be Gram-negative. This change in reaction is not infrequently observed in old cultures when dead and degenerating bacteria are present; under such conditions both Gram-positive and -negative forms may be observed in the same field. Faulty technique is frequently responsible for irregular results and the student must be careful to avoid this possibility.

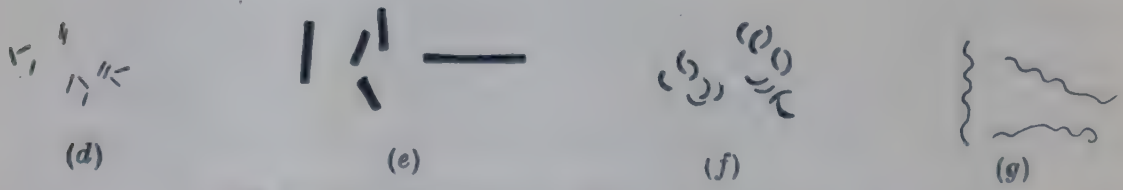
The reason for the different behaviour of the various bacteria when stained by the Gram technique is not properly understood. Some workers consider that a physical process is involved in the reaction and that the behaviour of an organism depends on the permeability of its cell-membrane to the stains. In the case of Gram-positive bacteria the iodine assists the penetration of the cell-protoplasm by the violet dye, whereas with Gram-negative organisms it has no such action. Others accept a chemical explanation, which postulates that in the case of Gram-positive forms the iodine links the dye to the protoplasm. Notwithstanding the lack of unanimity on the mechanism of the staining process, the importance of the Gram stain in the study of bacteria cannot be over-estimated.

Another important differential process is that described independently by Ziehl and Neelsen and generally known as the Ziehl-Neelsen method. Certain organisms, such as the tubercle bacillus, do not stain readily by the methods already mentioned ; they require prolonged exposure to the dye, frequently with the application of heat, but, when stained, they are resistant to the decolorizing action of acids. They have consequently been termed " acid-fast " bacteria. In some instances they resist also decolorization by alcohol ; these have therefore been called " acid- and alcohol-fast " bacteria. This increased resistance to dyes is considered to be due to the presence of some lipoidal or waxy substance in the cell.

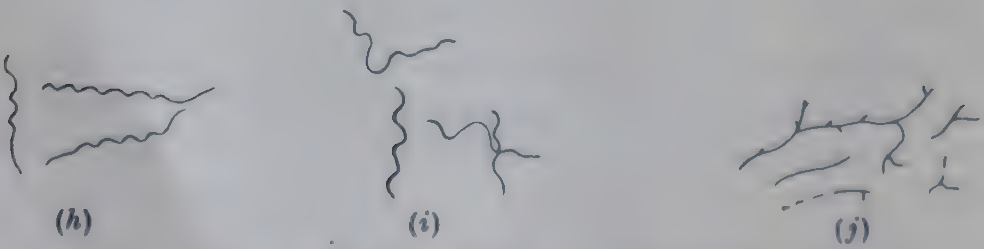
In carrying out the Ziehl-Neelsen stain, the film is covered



Cocci arranged (*a*) in clusters, (*b*) in chains and (*c*) in groups of four.



(*d*) and (*e*)—Bacilli, (*f*)—Vibrios, (*g*)—Spirilla.



(*h*) and (*i*)—Spirochætes, (*j*)—Actinomyces.

Fig. 2.

with carbol-fuchsin and heated, until steam rises, for 5 minutes, washed with water and decolorized with 15–25 per cent. H_2SO_4; it is then washed with water and counterstained with a weak aqueous solution of methylene-blue. The acid-fast bacilli stain a bright red colour, while the other bacteria and cells stain blue.

In the examination of spirochætes, which usually stain with difficulty, more complicated methods are frequently necessary; the methods used are those of Fontana, Giemsa and Levaditi.

By observations of unstained material together with the use of simple and complex staining methods, it is possible to examine in detail the structure and form of the different bacteria and obtain useful information about their probable identity. The following points should receive special attention.

Shape of Bacteria. Variations in the appearance of different bacteria were recognized by the earliest observers. Leeuwenhoek (1683) described clearly bacillary and spiral forms, while frequent attempts were later made to classify the bacteria on morphological appearances. These classifications are now of little value, but it is interesting to note that many of the terms employed in these early schemes are still in use. The main forms now recognized are the coccus, bacillus, vibrio, spirillum, actinomyces and spirochæte (Fig. 2).

A **Coccus** is a spherical form; in some cases, such as the pneumococcus, one diameter is definitely longer than the other, so that the form is oval rather than round. In others characteristic arrangements, such as long chains, pairs or small clusters which are produced mainly by the mode of division, are found.

A **Bacillus** is a cylindrical rod; great variation is found among the different members in the proportion of length to transverse diameter. Division always takes place by transverse fission after previous elongation and constriction at the site of the division. The form is variable and may be a straight rod with square ends, or the long axis may be bent and the ends rounded or pointed. The size of the different bacilli varies considerably; some are very large while others approximate to the coccal forms and are sometimes referred to as "cocco-bacilli". The term *Bacillus* has now been adopted as the generic name for a group of Gram-positive, sporing, aerobic, rod-shaped organisms, while the term *Bacterium* is used as the generic name of a group of Gram-negative rod-shaped forms. This is perhaps unfortunate, as there must consequently arise a certain amount of confusion from the use of these two terms, each of which has both a specific and a general application. However, when used in the generic sense these names are given a capital and are printed in italics.

A **Vibrio** is a cylindrical form slightly bent or curved on itself; a single cell may frequently present the shape of a comma.

A **Spirillum** is a form closely related to the vibrio and is characterized by a number of bends or spirals along the long axis.

An **Actinomyces** is a form differing from the above in that filaments and branching are found. These forms are sometimes referred to as the higher bacteria.

A **Spirochæte** is an elongated, flexible organism twisted spirally around its long axis and exhibiting motility without possessing flagella. It resembles in some respects both the bacteria and the protozoa.

These basic forms, under favourable conditions, tend to remain constant. It appears to be impossible to convert permanently one form into another, but under certain conditions, such as in old cultures, the morphology of certain organisms may show considerable variation. In the case of the diphtheria bacillus this morphological variation or *pleomorphism* occurs in young cultures and is a characteristic feature of the organism; in one field of the microscope the bacilli may be either regular thin and bent, or short and thick, or considerably enlarged at one end, *i.e.*, club-shaped.

The Size of Bacteria. Bacteria, as previously stated, are exceedingly minute bodies and represent one of the lowest orders of living things. The determination of the size of the different forms was originally carried out by comparison with known red blood corpuscles. A more accurate estimation is now obtained by the use of a special micrometer eye-piece, containing a graduated scale, which is first gauged by comparison with a standard graduated micrometer slide. The unit employed is a micron, μ, which is $\frac{1}{1000}$ of a millimetre or $\frac{1}{25000}$ of an inch.

There is naturally a great variation in the size of the different bacterial forms. Considerable irregularity may also be found in different members of the same species, the size of a bacterium being largely dependent on environmental conditions. Among the coccal forms the average diameter varies from around $0{\cdot}75\mu$ in the case of the staphylococcus to $1\text{–}2\mu$ with the meningococcus. The rod-shaped organisms exhibit a particularly wide dimensional range, *e.g.*, the anthrax bacillus is usually $6\text{–}12 \times 0{\cdot}7$–

$1{\cdot}5\mu$, the influenza bacillus $1{\cdot}2 \times 0{\cdot}3\mu$, while some species may develop filamentous forms exceeding 80–100μ in length.

Under the most favourable conditions the limit of resolution by direct microscopy is approximately $0{\cdot}2\mu$; under the usual working conditions it is probably considerably more than this (about $0{\cdot}275\mu$). This is not, however, the limit of the size of living bodies. It is now accepted that many important diseases of an infectious nature occurring in man and animals are caused by particulate bodies with dimensions frequently much less than $0{\cdot}2\mu$ in their greatest diameter. These bodies are termed *filterable viruses* or more simply *viruses*.

The Arrangement of Bacteria. As a result of sub-division and slow or incomplete separation of the daughter-cells some bacteria tend to arrange themselves in characteristic aggregations. A wide variety of arrangements is shown by the cocci, as they may divide in any plane; the other forms are more restricted in this respect, as they always divide in the transverse diameter. Some cocci divide in one direction only and chain-formation is observed, as in the case of the streptococci; in other instances the two cells resulting from the primary division form a diplococcus; some cells divide in any axis and form irregular groups or clusters of cocci, which resemble bunches of grapes, *e.g.*, the staphylococci. Certain cocci divide in two planes, one at right angles to the other, forming tetrads, and others in three planes, producing cubes of eight organisms, as in the case of the sarcinae.

Some bacilli may also present peculiar arrangements, which are often characteristic; adjacent diphtheria bacilli frequently lie at an angle to one another, forming V or L shapes, while groups of the bacilli may resemble Chinese letters or pallisades.

The Bacterial Cell. Bacteria are composed mainly of a homogeneous colourless, smooth protoplasm which is contained within a fine delicate membrane or envelope. This membrane determines the contour of the cell, but it is difficult to demonstrate as it stains in a similar manner to the cytoplasm. Its presence is suggested by the results of plasmolysis, which have been obtained with a few of the larger organisms. When these bacteria are placed in a hypertonic salt solution the cell-fluid passes out, retraction of the cytoplasm occurs and the outline of a cell-membrane is exposed. This membrane appears to be a definite differentiated part of the cell.

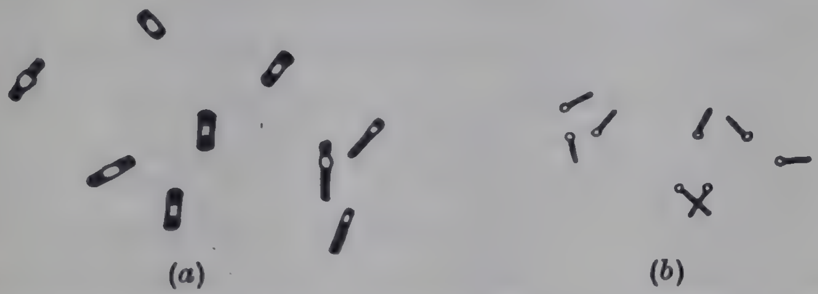
The presence of a *nucleus* is a controversial question which still remains unsettled. No satisfactory demonstration of a true nucleus in a bacterium has been made; it is possible that this failure is due to the inherent technical difficulties and the limitations of the microscope. It appears highly probable that some nuclear apparatus is present, and the general opinion is that the nuclear material, chromatin and the protoplasm are mixed together in the bacterial cell. Thus an organized nucleus has not developed and only a primitive form exists.

In certain bacterial species, *e.g.*, the diphtheria bacillus, granules, which stain deeper than the rest of the protoplasm, are present. These have been termed *metachromatic granules*, *volutin granules* and *Babes-Ernst bodies;* in some cases these bodies occur at the poles of the bacterium and are called polar bodies. The affinity of these bodies for the basic dyes resembles that of chromatin and is explained by their content of nucleo-protein. They are not, however, considered to represent nuclear structures, but are probably reserve food substances. Special staining methods have been introduced to demonstrate these bodies.

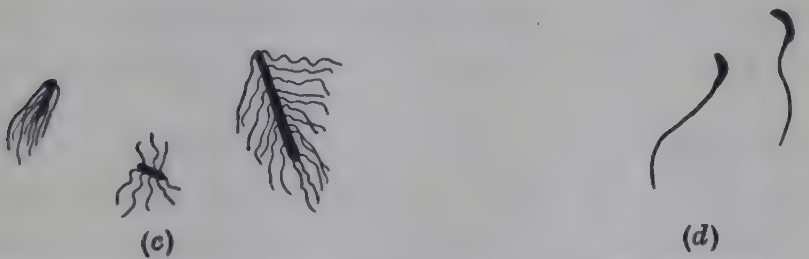
The acid-fast bacilli contain in their cytoplasm a lipoidal substance, which is probably of the nature of a higher alcohol. Fat particles, glycogen and starch granules have been demonstrated by appropriate staining methods in many bacterial species.

Capsules. The presence around some bacteria of a mucoid or gelatinous capsule can be demonstrated by suitable staining methods. Many workers consider that all bacteria possess a capsule, but that in only a few cases is this sufficiently large to be observed. It is interesting to note that capsules have been definitely demonstrated on certain virulent bacteria, *e.g.*, *Strept. hæmolyticus*, which were, at one time, considered to be non-capsulated. Bacteria having a readily demonstrable capsule, such as the pneumococcus and Friedlander's bacillus, are frequently referred to as " capsulated " bacteria. It is, however, important to note that such organisms do not always show this capsule, its presence depends largely on the environment in which growth is taking place. Capsule-formation can be readily observed in organisms growing either in the animal tissues or, when recently isolated, in artificial media freshly prepared and containing some animal protein, such as blood, serum or ascitic fluid. The capacity to produce capsules becomes less and less pronounced on repeated subculture until finally it is lost.

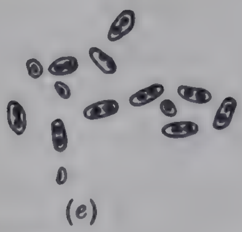
The origin of the capsular substance is uncertain, but it probably results from some change in the outer layer of the cell-membrane. In many cases it has been found to contain a complex polysaccharide and to be concerned with the specificity of the organisms, *e g.*, the pneumococcus. The precise function of the capsule is unknown, but there is much evidence to indicate that its presence is directly related to the virulence of the organism. Encapsulated organisms are able to resist phagocytosis.



Spores—(*a*) central and subterminal ; (*b*) terminal (*Cl. tetani*).



Flagella—(*c*) peritrichate ; (*d*) monotrichate (*V. choleræ*).



(*e*) Capsulated cocci (pneumococcus).

Fig. 3.

Capsules (Fig. 3) are not stained by the ordinary staining methods, by which the cell-bodies frequently appear to be surrounded by an unstained halo. They can, however, be demonstrated by such special methods as those of Muir and Hiss.

Spore-Formation. Under certain conditions there develop within the protoplasm of some bacterial cells oval or spherical bodies which are difficult to stain. These bodies are called *spores* or *endospores* and occur mainly among the Gram-positive bacilli ; cocci never form spores. The presence of spores was described by Cohn in 1875 and later by Koch in 1876, but the precise method of their formation is still unsolved. There appears to be a localization of a chromatin-like substance which becomes surrounded by a thick, resistant membrane. As the spore develops the mother-cell degenerates and finally disappears, leaving the free spore.

The position of the spore may be either central (equatorial), subterminal or terminal, and its shape spherical or oval (Fig. 3). The size varies and in some cases the diameter of a mature spore may be greater than that of the bacterial cell, the length of which is always greater than that of the spore. Characteristic appearances are sometimes found, such as the "drum-stick" form given by those bacilli with a large spherical, terminal spore, *e.g.*, the tetanus bacillus. Spores are resistant not only to staining, special methods being required, but also to unfavourable surroundings and destructive agents. This is due both to the relatively thick and impermeable membrane which encloses the protoplasm and the low water-concentration of the protoplasm.

The formation of spores is not a method of multiplication ; almost invariably one organism forms one spore, which in its turn develops into one organism. The significance of sporulation is uncertain, but it is generally considered to be a resting phase occurring when the environmental conditions, particularly the food supply, are unfavourable for vegetative growth. When placed in conditions suitable for growth the spore germinates by rupture of the membrane and the ordinary or vegetative form of the organism is produced. It is interesting to note that in many cases sporulation only occurs at temperatures especially favourable for growth and that, in the case of organisms requiring the presence of free oxygen for growth, spore-formation does not occur in the absence of this gas.

Flagella. Many bacteria exhibit active motility ; this, except in the case of the spirochætes, is associated with the presence of thin, delicate, long threads termed *flagella* which are exceedingly difficult to stain, special methods being required. The flagella of different bacteria vary not only in length, but also in the position at which they join the bacterial cell (Fig. 3). A classification of the bacteria has been formulated on this basis :—

(1) "Monotrichate" bacteria having a single flagellum at one pole.

(2) "Lophotrichate" bacteria having a tuft of flagella at one or both poles.

(3) "Amphitrichate" bacteria having a single flagellum at both poles.

(4) "Peritrichate" bacteria with flagella completely surrounding the body.

Motility is most conveniently detected by the use of the hanging-drop technique, and is generally demonstrated with most certainty in young cultures grown at the optimum temperature for the bacterium under examination. Care must, however, be taken to distinguish true motility from Brownian or molecular movement, which is due to a surface bombardment by molecules in the fluid. Brownian movement is a rapid vacillation of the particles without any definite progressive movement from one place to another. It is also necessary to exclude any movement due to the presence of currents in the drop, in which case all organisms move in the same direction. Motility is only exhibited by the cocci in extremely rare instances, but it may be shown by all the other bacterial forms. It is important to note that while motile bacteria may become non-motile, non-motile bacteria never, at any stage of their development, exhibit motility. Motility may be lost by various procedures, *e.g.*, leaving cultures at room-temperature, prolonged incubation or cultivation on unsuitable media.

The origin and nature of the flagella are uncertain; some workers consider that they arise from the capsule and others from the cell-protoplasm. It has, however, been conclusively demonstrated that they are antigenically distinct from the cell-bodies.

Reproduction of Bacteria. Bacteria multiply by division or simple fission, each organism producing two daughter-cells; there appears to be no true sexual phase. Division takes place at right angles to the long axis in the case of the cylindrical forms; cocci tend to become elliptical immediately prior to division and the direction of cleavage governs to a large extent the subsequent arrangement of the bacteria. In many cases after cleavage the young forms are coherent and remain so until they approximate the adult size. Mitotic changes in the cytoplasm have not been found during the division. Multiplication can be readily observed in a hanging-drop preparation. The rate of division varies with the environmental conditions; under optimal conditions for growth it has been found that the life of one generation, *i.e.*, from one cleavage to the next, may be as short as 15–20 minutes. Under favourable conditions a single bacterium may thus produce millions of similar cells in 24 hours. When the environmental conditions are less satisfactory, as in old cultures, multiplication is difficult and slow ; while changes in the form of the bacteria are also frequently observed. The organisms tend to become swollen, granular and less readily stainable ; these abnormal cells are termed *degeneration* or *involution* forms. They are often no longer viable, and when transferred to a suitable medium growth is usually slight or absent.

CHAPTER III

THE BIOLOGY OF BACTERIA

Bacteria are usually present wherever there is dead organic material; they are consequently widely distributed in nature, being found on the surfaces of the body, in soil, dust, water, manure and the air. The majority of bacteria are not restricted in their distribution, as they are able to survive and multiply under very simple conditions; others, however, can only grow under special circumstances and their presence in any vicinity is governed by the local conditions. Bacteria have been classified according to their growth requirements into two indefinite groups, but this arrangement is far from satisfactory. Bacteria that normally grow at the expense of dead organic matter have been termed *saprophytes*. A relatively small number of bacteria multiply under natural conditions only in the body-tissues and have been called *parasites*. These terms are, however, only relative; it is impossible absolutely to separate one group from the other. Under some conditions a parasitic organism may lead a saprophytic existence and *vice versâ*. Some bacteria, such as the gonococcus and the influenza bacillus, are strict parasites and multiply in nature only in the tissues of the host; other so-called parasites are, however, found not infrequently growing under saprophytic conditions, and some bacteria, which usually multiply on dead material, are able to grow in the body-tissues. Growth in practically all cases can be obtained *in vitro* if a satisfactory culture medium is employed.

Chemical Composition of Bacteria. Attempts have been made to analyse the chemical constitution of various bacteria in order to throw some light on their biological activity. The results at present available are very incomplete, but there is little doubt that this question will receive more attention in the future. The composition of bacteria is of great importance in the study of immunity; many forms possess a complex antigenic structure, the components of which are chemically distinct. Different, but usually related, species, however, not infrequently possess a common antigen.

The data at present available indicate that bacteria are composed of water, proteins, lipoids, carbohydrates and salts. The ratio of these substances varies over a wide range not only with the different bacteria, but also with the same organism under different environmental conditions. The age of the culture, the composition of the culture-medium and the conditions of growth are some factors influencing the composition of bacteria. Much (80–90 per cent.) of the protoplasm is water, while some 50–80 per cent. of the dried residue is protein, part of which has been found to be nucleic acid and its products. The salts are mainly the phosphates, sulphates and chlorides of potassium, sodium, calcium and magnesium. Some bacteria also possess waxes, higher alcohols and pigments.

Osmosis. The bacterial cytoplasm is contained within a delicate membrane. This behaves in many ways as a semi-permeable membrane, in that it allows water to pass through but exhibits individual differences in its permeability by salts in solution. This phenomenon of diffusion by the solvent, but not solute, through a semi-permeable membrane is termed *osmosis*. The method of demonstrating the cell-membrane by *plasmolysis*, *i.e.*, by placing the bacteria in a hypertonic salt solution, is an illustration of the osmotic activity of the bacteria. The opposite phenomenon, *plasmoptysis*, may be observed on placing bacteria in distilled water or a hypotonic salt solution; water passes into the bacterial cells, which swell and may finally burst. Bacteria are usually isotonic with physiological saline, which is therefore generally employed in the preparation of bacterial suspensions. The osmotic behaviour of the different bacterial species varies greatly, but in most cases the egress or ingress of water is slow. It is interesting to note that the phenomenon of *plasmolysis* has only been demonstrated with a few large saprophytic organisms.

Enzymes. Substances which initiate and accelerate chemical reactions without themselves being chemically changed are termed *catalysts*. Active catalysts are produced by living cells, and they are usually termed *enzymes* or *ferments*. A number of bacteria have many different enzyme actions, in some cases 50—100; the distribution of the enzymes in the bacterial cell is, however, unknown. They do not always remain intracellular, but may escape into the medium in which they are growing. The metabolism of bacteria is controlled to a large degree by the activity of the different enzymes they produce. It has been found that the enzymic constitution of bacteria may be changed by adapting organisms to grow in media of special composition; this is a change in response to the stimulus of chemical environment and is doubtless the factor mainly responsible for the adaptation of certain pathogenic bacteria to cultivation in relatively simple media. It has been suggested that bacterial enzymes should be divided into two classes—(1) *constitutive enzymes* which are invariably present in the bacterial cell, and (2) *adaptive enzymes* which can be developed by growth on a medium containing the corresponding substrate.

Bacterial enzymes act in numerous ways and are usually given the name of the substance acted on plus the suffix "*-ase*"; thus the enzyme, *lactase*, splits lactose into glucose and galactose, *proteases* act on proteins and *lipases* split fats into fatty acids and glycerol. These effects are brought about by hydrolysis.

Enzymes play an important *rôle* in nature; complicated organic matter is broken down by their activity into a form which can be utilized by bacteria, plants, etc., as an adequate food supply. Synthesis of these relatively simple products into the complex cell protoplasm is again the result of enzyme activity.

Enzymes are relatively labile substances; they are inactivated at 56°–60° C. in half an hour, and are inhibited and often destroyed by the action of strong acids and alkalies, but are resistant to freezing; their activity is accelerated within certain limits by raising the temperature of the reaction. An enzyme reaction is, as a rule, reversible and exhibits a high degree of specificity.

Food Requirements of Bacteria. The food requirements of the different bacteria exhibit a remarkably wide range of variation. Water is essential for growth; while the amount required by individual bacteria varies considerably, it is an accepted fact that multiplication cannot take place on an absolutely dry surface. The incomplete results obtained by the chemical analysis of bacteria indicate that the following elements are required to build up their complicated protoplasm: oxygen, hydrogen, nitrogen, carbon, sulphur, phosphorus, together with salts of calcium, potassium, magnesium, sodium and iron.

The needs of many saprophytic bacteria, such as those found in the soil, are extremely simple, growth taking place without difficulty. The parasitic bacteria are usually more exacting, and frequently only multiply in media containing complex organic material; some, *e.g.*, the gonococcus, only grow on first isolation in the presence of some animal protein such as serum, ascitic fluid or blood. It is thus impossible to generalize on bacterial metabolism.

The nutritive requirements of bacteria have been examined in great detail by the use of synthetic media containing only known chemical compounds. Knight carried out an extensive investigation into the problem and found that the development of nutritional requirements could be divided into four main stages. In the case of the true *autotrophic* bacteria, *e.g.*, the nitrifying bacteria of the soil, the requirements are very simple; carbon is assimilated as CO_2, and nitrogen obtained from inorganic sources, especially ammonia. This represents the first stage of nutritional evolution; these organisms must possess very marked synthetic activities as their protoplasm is apparently similar in general structure to that found in other bacteria. The requirements become increasingly complex through stages two and three and, in the fourth stage, energy and carbon are obtained from organic compounds, while nitrogen must be present in the form of amino-acids; accessory and growth factors may also be necessary. These organisms are termed *heterotrophic* bacteria and exhibit a wide variation in synthetic power, which individually may be very limited. The marked increase in the complexity of nutrient requirement is considered by Knight to be closely related to the pathogenicity of these organisms, and also to represent the course of bacterial evolution. The tissues of the host provide special components for the synthesis of protoplasm and the power to produce these is gradually lost by the parasitic bacteria. Restriction of synthetic activity leads eventually to strict parasitism, when the necessary growth requirements can be supplied only by living tissues as in the case of the viruses.

Fildes considers that the synthesis of bacterial protoplasm is carried out in progressive stages of enzymic activity; at each of the stages the chemical group forming an essential link in the chain of synthesis is referred to as an *essential metabolite*. In many instances the essential metabolites are synthesized by the bacteria themselves but where this does not occur, it is necessary to add these substances to the medium, otherwise growth does not take place. The essential metabolite is then referred to as a *growth factor*. These points can be illustrated by the behaviour of the following organisms—*Bact. coli* can reproduce itself from ammonia but *Bact. typhosum* only grows if amino-acids are also added and the *Staphylococcus* if nicotinic acid is present. Amino-acids and nicotinic acid are thus essential metabolites for all three bacteria, but nicotinic acid is a growth factor only for the *Staphylococcus* and amino-acids only for *Bact. typhosum*, as these vital substances cannot be synthesized by the respective organisms.

Bacteria, as a rule, are very adaptable and are able to grow on a wide variety of media. In the case of certain essentially parasitic organisms, such as the gonococcus and the meningococcus, growth takes place only on enriched media when freshly isolated from the body-tissues. However, after culture on artificial media for some time growth can usually be obtained on simpler media. In view of the great variation in the requirements of the different bacteria, it is in no way surprising that a large number of media has been described (see Chapter XXXVII.).

In addition to the food supply other factors exercise a great influence on bacterial multiplication ; important in this respect are the atmospheric conditions, the temperature of incubation and, in certain cases, the presence of accessory factors in the medium.

Atmospheric Conditions. Considerable variation is shown by the different bacterial species in their atmospheric requirements. All, however, multiply better in the dark than in the light ; direct sunlight generally exercises a marked bactericidal action, which is due mainly to the ultra-violet rays.

Respiration is exhibited by all bacteria, oxygen exchange being an essential feature of bacterial metabolism. Great variability in oxygen requirement is shown by the different species. Many obtain their oxygen from the air and grow best under ordinary atmospheric conditions ; these are termed *aerobes*. Other species, however, are unable to reproduce in the presence of molecular O_2, any oxygen required being obtained by splitting up the complex proteins and carbohydrates in the medium ; these are termed *anaerobes*. A large number of aerobic bacteria are also able to grow in the absence of free oxygen and are sometimes called *facultative anaerobes*. Anaerobic bacteria, the growth of which is inhibited by minute traces of free oxygen, are referred to as *obligate anaerobes*. Some bacteria grow most luxuriantly under modified atmospheric conditions ; oxygen is required, but less than is present in the air ; these organisms are termed *micro-aerophiles*. There is no sharp line of demarcation between aerobes and anaerobes. Many aerobes are able to multiply under anaerobic conditions, while even strict anaerobes can grow under ordinary atmospheric conditions if strongly reducing substances, such as cystein or pieces of tissue, are added to the medium.

It has recently been shown that the presence of CO_2 in a medium is necessary for the growth of many bacteria. In the majority of cases the required amount of CO_2 is produced by the bacteria of the inoculum and there is no need to make provision for a special supply. Some organisms, however, particularly on first isolation on artificial media, require the presence of additional CO_2; *e.g.*, the gonococcus and *Br. abortus* grow on first isolation most satisfactorily in the presence of 10 per cent. of CO_2.

The explanation of the various oxygen requirements of the different bacteria is uncertain. It has been suggested that, in the case of the anaerobes, the atmospheric oxygen does not itself exert an injurious action on the organisms, but that it is used up during bacterial growth to form hydrogen peroxide, to which these organisms are very susceptible. The aerobes, on the other hand, are resistant to H_2O_2, as in the case of the pneumococcus, or if susceptible produce a catalase, which breaks it down into water and oxygen. Although this theory is very plausible, it does not explain all the associated phenomena, *e.g.*, *Cl. sporogenes* (an anaerobe) is resistant to H_2O_2, while a few anaerobes produce abundant catalase. Another hypothesis is that a limiting oxidation-reduction potential is necessary for the growth of the anaerobes, and this is not obtained under atmospheric conditions with ordinary media.

Temperature. The bacteria, as other living bodies, are greatly influenced by the temperature to which they are subjected. Multiplication usually takes place within a given range, and beyond this growth is inhibited. For all species there are not only maximum and minimum temperatures but also one at which growth is most rapid and prolific, this is termed the *optimum temperature*. Above this multiplication occurs less readily until there is reached the maximum temperature for growth, beyond which destruction of bacteria takes place; this becomes more and more marked as the temperature increases. At temperatures below the optimum reproduction becomes slower until the minimum temperature for growth is reached. Below the minimum temperature some bacteria tend to die, but the majority remain dormant and enter on a resting phase.

The optimum temperature for the various bacterial species shows marked variation and depends largely on the habitual environment to which they are accustomed. In the case of human or animal parasitic bacteria the optimum temperature is generally about 37° C., *i.e.*, about the body temperature. On the other hand, many of the saprophytic organisms present in water and the soil multiply best at lower temperatures, 20°–25° C. Some organisms, which have been isolated from warm springs and manure, grow best at about 60° C.; these have been termed *thermophilic* bacteria.

In the case of most bacteria of medical importance, multiplication takes place over a wide range of temperature, 18°–42° C., but in a few instances, such as the influenza bacillus, the range is very limited, since the organisms are extremely susceptible to changes of temperature from 37° C.

Low temperatures are much less destructive than high ones. Growth does not usually take place below 10° C., although the author has isolated a bacillus which grew luxuriantly at 4° C. At zero many bacteria lie dormant, and consequently at this temperature they can conveniently be conserved. It has been found that diphtheria bacilli are able to withstand a temperature as low as — 200° C. An illustration of the absence of injurious effects of cold is that typhoid bacilli have not infrequently been isolated from ice-cream.

High temperatures are markedly destructive to bacteria, moist heat being more effective than dry heat. As the temperature is increased a point is reached when the bacteria are all killed; this is termed *the thermal death point.* It is important to note that the death point of a bacterium is not constant, but depends on a number of variable factors, important of which are the time of exposure, the menstruum and the number of bacteria present. The spore-bearing forms are most resistant to heat and temperatures over 100° C. may be required for their destruction.

Accessory Factors. In the case of a few strict parasites, *e.g.*, the influenza bacillus, growth only occurs when there are present in the medium certain substances which have been termed *accessory factors.* Their nature is uncertain, but they resemble the vitamins in some respects. They have been studied mainly in connection with the influenza bacillus, for the multiplication of which two accessory factors, termed "*X*" and "*V*," are essential. The "*X*" factor is thermostable, resisting a temperature of 120° C. for 45 minutes, present in blood and closely related to hæmatin. The "*V*" factor is relatively thermolabile, being inactivated at temperatures above 100° C.; it is present in blood, yeast and certain vegetables, such as the potato, and appears to behave as a co-enzyme.

Biochemical Activity. Bacteria are not only responsible for a number of important human and animal diseases, but they also play an essential *rôle* in nature by making possible the chemical interchange between the animal and vegetable kingdoms. This function is performed by the breaking down of the complex molecules by the bacterial enzymes. As a result energy is provided and simple substances, which can be assimilated by the organisms, are produced. In relatively few instances the opposite process occurs, that of building-up or synthesis. This activity is an important property of certain of the soil bacteria, which are able to fix and utilize the atmospheric nitrogen.

The process of fermentation was considered to be purely chemical until the ingenious and conclusive experiments of Pasteur, whereby the *rôle* played by the bacteria was decisively demonstrated. It is now recognized that a bacterium may possess many enzymic activities ; different sugars are fermented and various proteins and fats are broken down. In a number of cases these actions are reasonably constant under normal conditions and consequently biochemical tests may be of great value in establishing the identity of an organism. The possibility of modifying the enzymic activity of an organism by the process of adaptation has already been noted ; this, however, does not detract from the value of the tests in diagnosis.

Carbohydrate Metabolism. The splitting up of the complex carbohydrate molecules is termed *fermentation.* This term was originally applied to all forms of bacterial metabolism and was frequently employed to indicate disintegration of proteins, but it is now usually restricted to carbohydrate metabolism. The dissimilation of the complex molecule to the simple compounds, such as carbon dioxide, lactic acid and alcohol, is produced by yeasts, moulds and bacteria, and takes place in a number of successive stages. Fermentation by yeasts frequently results in the production of alcohol and, as yeasts are employed extensively in the manufacture of wines and beer, this reaction has been studied very closely.

The fermentation, or saccharolytic activity, exhibited by the different bacteria varies considerably. Some species possess a wide range of activity and split up complex polysaccharides, such as starch and dextrin, as well as the simpler sugars. Others, however, may attack only the simple monosaccharides or hexoses, and some the disaccharides, such as lactose, as well as the monosaccharides.

Many substances are produced by bacterial fermentation the most frequent end-products are lactic acid, propionic acid, formic acid, acetic acid and butyric acid. Gas is frequently formed and consists usually of CO_2 and H_2. Polysaccharides and disaccharides are first hydrolyzed to the simple hexoses before fermentation takes place.

Thus in the splitting up of a disaccharide, *e.g.*, lactose, by the enzyme *lactase*, the following reaction constitutes the first stage :—

$$\underset{\text{lactose}}{C_{12}H_{22}O_{11}} \underset{+}{+} \underset{\text{water}}{H_2O} = \underset{\text{glucose}}{C_6H_{12}O_6} \underset{+}{+} \underset{\text{galactose.}}{C_6H_{12}O_6}.$$

In the splitting up of the hexoses the production of gas depends on the activity of the enzyme involved. Thus *Bact. typhosum* acts on glucose with the production of formic acid, at which stage fermentation ceases. When, however, *Bact. coli* acts on the same sugar, the process goes a step further and the end-products are CO_2 and H_2 ; *Bact. coli* forms an enzyme which can break down formic acid in the following manner :

$$H.COOH = CO_2 + H_2.$$

For the diagnostic application of the fermentation tests various sugar solutions of 0·5–1 per cent. strength are made in peptone-water or serum-water medium. A suitable indicator is also added and the activity of the bacteria is indicated by the production of acid with, or without, gas formation.

The following are the carbohydrates in frequent use :—

(*a*) **Monosaccharides**—glucose, arabinose, xylose, lævulose and galactose.

(*b*) **Disaccharides**—lactose, maltose and sucrose (saccharose).

(*c*) **Polysaccharides**—starch, inulin, dextrin and glycogen.

Other related substances are also used in diagnosis, *e.g.*, complex alcohols such as glycerine, dulcite, mannite and sorbite, and glucosides, such as salicin.

Protein Metabolism. Some bacteria, such as *B. subtilis*, *Cl. sporogenes*, and *Proteus vulgaris*, have a marked proteolytic activity. The breaking down of proteins by bacteria is usually termed *putrefaction*. Some, however, restrict this term to the decomposition of proteins under anaerobic conditions, when, in the absence of O_2, the end-products are amino-acids, indole, H_2S and mercaptans, which cause the particularly foul odour ; the gases formed are largely H_2S, CO_2 and H_2. Proteolysis occurring under aerobic conditions tends to be more complete and is termed *decay*. The end-products here are amino-acids, indole and H_2S ; mercaptans are never formed, and the odour is consequently less offensive than that resulting from putrefaction. In nature putrefaction and decay usually occur side by side.

All types of albuminous material may be attacked by bacteria; many bacteria will not grow in the presence of pure proteins alone and simpler breakdown products should be provided in the media. In the laboratory indications of proteolytic activity are usually obtained by the power of the various bacteria to liquefy gelatin, fibrin or coagulated serum, to produce indole or H_2S, and to digest milk and meat.

The enzymes responsible for protein-cleavage are termed *proteases*. The process is usually, in the first stages, a simple hydrolytic cleavage of the complex protein molecule as a result of which proteoses, peptones and amino-acids are produced. These may be split up to ammonia and fatty acids and the latter may be further decomposed into CO_2, H_2 and methane. Proteolytic activity supplies bacteria with their main source of nitrogen.

The bacterial proteases have many properties similar to those possessed by trypsin, pepsin and erepsin, which are concerned in the human digestive processes. An exact study of the various processes involved is, however, extremely difficult, owing to the complex media in which the reactions take place.

The importance of bacteria in the breaking up of proteins is illustrated in the modern methods of meat preservation. By the conservation of meat in refrigerators at temperatures around zero, bacterial growth is inhibited and, as a result, decomposition of the meat does not take place. Organisms capable of multiplying at low temperatures may, however, prove troublesome.

Fat Metabolism. The enzymes responsible for fat-cleavage or lipolytic activity are termed *lipases*. This process has not received universal attention, but it is of great importance in the preparation of butter and cheese, and is probably hydrolytic in nature.

Pigment Production. Many bacteria are able to form a characteristic pigment; these are termed *chromogenic* bacteria. The type of pigment varies considerably with different species and may be yellow, red, violet, green, etc. In some cases it remains within the bacterial cell, while in others, *e.g.*, *Ps. pyocyanea*, it diffuses into the surrounding media, *i.e.*, it is extracellular and water soluble. The production of pigment is dependent to a considerable degree on environmental factors. Aerobic conditions are usually essential, but in the case of *Sp. rubrum* pigment-formation is most marked under reduced oxygen pressure. Pigment production is facilitated by growth at relatively low temperatures; in many cases it is formed more readily on incubation at between 20°–30° C. than at 37° C. Certain media, such as the potato, favour pigment formation, which is usually more marked by a freshly isolated organism than by one conserved on artificial media for some time.

Little is known about the exact chemical nature of the different pigments; in only a few cases has the constitution been determined. Pigments have, however, been divided chemically into three main types:—

(1) *Carotenoid* pigments are red, orange or yellow, and are soluble in ether, alcohol and chloroform.

(2) The *Anthocyanins* are the red to blue pigments and are soluble in water and alcohol but not in ether.

(3) The *Melanins* are the black pigments and are very insoluble.

The main practical importance of pigment production is that it may provide useful information in the identification of an organism.

CHAPTER IV

THE CULTIVATION OF BACTERIA

ALTHOUGH most bacteria are able to multiply in nature under a large variety of conditions, a detailed study of their activity is only possible by the examination of the growth characteristics under artificial conditions. In the majority of instances the needs of the bacteria are not very specialized, but, on the other hand, some parasitic members are very exacting in their requirements. Considerable adaptability is, however, displayed by most organisms ; on first isolation from the tissues some grow very poorly, but, after several subcultures, they gradually adapt themselves to the new conditions and growth becomes more marked and occurs with a wider range of media. The technical methods employed in the cultivation of bacteria have evolved gradually from those originally introduced by Koch. Innumerable media have been described, but it is impossible to be acquainted with all ; details of those in general use are given later (Chapter XXXVII.).

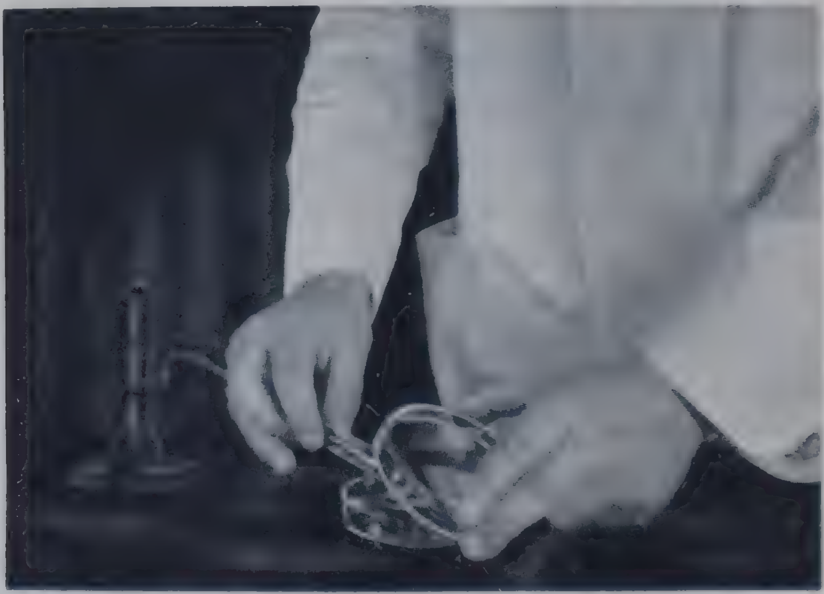


FIG. 4.—Petri dish and the method of pouring a plate.

The media are contained in suitable receptacles, which are made of glass to facilitate the observation of the results of growth. The glassware is usually employed in the form of test-tubes, screw-cap bottles, flasks or Petri dishes, and must be thoroughly cleansed before use. A Petri dish or plate is composed of two circular dishes, one of which acts as a lid; the diameter of the base is usually 9 cm. (Fig. 4).

Culture media are divided into two main types: (1) fluid, and (2) solid. In some cases, as in the cultivation of spirochætes, a third type, the semi-solid media, is frequently used.

Fluid Media. The early studies of the *rôle* played by bacteria in spontaneous generation and fermentation were carried out by observations of their activity in fluid media. This method was both tedious and inaccurate as, by the use of a fluid medium alone, pure cultures, *i.e.*, cultures of one species only, are extremely difficult to obtain. The introduction of a solid medium by Koch was a step of the greatest importance in the isolation of pure cultures and remarkable advances have since been made. Nevertheless fluid media serve a useful purpose, and they are used extensively in the study of pure cultures.

The food requirements of the bacteria have already been discussed, and it has been noted that the following elements are necessary for the synthesis of the bacterial protoplasm: carbon, oxygen, nitrogen, hydrogen, in addition to a number of mineral salts. These substances form much of the soluble constituents of meat, and meat extract is consequently utilized as the basis of many media.

Preparation of Meat Extract. Lean beef or heart muscle of the ox is freed from fat and finely minced. To 1 lb. of the minced meat add 1,000 c.c. of water, mix and allow to stand in a cool place for 24 hours. Then strain through muslin and boil for 2 hours to coagulate the heat-coagulable proteins; filter through paper and bring the volume back to 1,000 c.c. with water. This extract is kept in a large flask until required. Commercial meat extracts, such as Liebig's beef extract, may be used instead of fresh meat, but the results are generally not so satisfactory.

The meat extract alone is not suitable for the growth of bacteria. Although it contains mineral salts and extractives it is lacking in nitrogenous material, as the meat proteins are coagulated by the boiling process and removed by the subsequent filtration. Digested and uncoagulable protein is therefore added in the form of a commercial peptone, which contains peptone and albumoses; this addition should be made before boiling. A similar result may be obtained by the partial digestion of the minced meat with trypsin.

Broth or Bouillon. A considerable number of media are prepared from the meat extract; the one most widely used is meat extract broth or bouillon. Salt and peptone are added to the extract in the following proportions :—

Meat extract	1,000 c.c.
Sodium chloride	5 gm.
Peptone	10 gm.

Boil to dissolve the ingredients and make slightly alkaline to litmus; filter through filter paper and make up to the original volume with water; place in flasks plugged with wool and sterilize. When required the broth is run into sterile test-tubes in amounts of 5–10 c.c.

Adjustment of the Reaction. The reaction of a medium is a matter of great importance owing to the varied requirements of different bacteria, and before use the reaction of all media is adjusted to a given *p*H. The term "*p*H" is employed extensively in bacteriology and is an index of the H ion concentration, *i.e.*, the reaction, of a solution. *p*H is the logarithm of the reciprocal of the hydrogen ion concentration; this relationship can be illustrated in the following manner :—

$$pH = \log I/\text{H ion concentration.}$$
$$\text{H ion concentration of distilled water} = 1/10^7.$$
$$\therefore pH \text{ of distilled water} = 7.$$

Distilled water is neutral in reaction as the molecule H_2O dissociates into one hydrogen ion (H) and one hydroxyl ion (OH). A *p*H of 7 is thus indicative of neutrality, a *p*H over 7 alkalinity, and one below 7 acidity. The majority of bacteria grow best in slightly alkaline media at a *p*H of 7·2–7·6.

The estimation of *p*H may be carried out very roughly by means of litmus paper, but, as in many instances the parasitic bacteria only multiply within a limited range of reaction, the adjustment of the medium has consequently to be more accurately performed. It is then carried out by comparing the reaction of the medium by means of an indicator, such as phenol-red ($1/10^4$), with that given by a prepared standard. Phenol-red exhibits a definite range of colours from yellow to purplish-pink through a *p*H range of 6·8 to 8·4. Solutions of known hydrogen-ion concentration are prepared to form a range of *p*H 6·8–8·4 with intervals of *p*H 0·2, and to each a fixed amount of phenol-red is added; these form the standard range. Phenol-red is added to a given quantity of the medium (5–10 c.c.), and by comparing the colour with the standard scale the pH of the medium is determined. The required reaction is then obtained by the addition of N/10 HCl or N/10 NaOH to the medium to render it either acid or alkaline respectively. The medium is adjusted in 5–10 c.c. amounts until the desired pH is obtained, the total quantity of HCl or NaOH required to adjust the bulk is then readily calculated.

Certain bacteria are very sensitive to changes in the reaction of the medium in which they are growing, and unless precautions are taken growth may be prematurely arrested. It is therefore essential that alterations in the reaction of the medium by substances produced as a result of the activity of the organisms should be avoided. In such cases this possibility is reduced by the addition of substances that tend to stabilize the reaction of a solution by taking up any dissociated H or OH ions. These substances are termed *buffers*; those in frequent use in bacteriology are the phosphates, citrates and carbonates of sodium and potassium.

Peptone Water. Another common medium, which is frequently used both alone and as a basis for more elaborate media, is peptone water; this is readily prepared :—

Peptone	1 gm.
NaCl	0·5 gm.
Water	100 c.c.

Peptone and the salt are dissolved in water, which is afterwards filtered. It is frequently used in testing bacteria for their capacity to produce indole and also as a basis for the various sugar solutions in the fermentation tests.

Solid Media. Solid media are usually prepared by the addition to broth of some substance such as gelatin or agar, which gives consistency to the medium.

Gelatin, an albuminoid derived from tendons and cartilage, was the substance originally employed by Koch in the preparation of solid medium. It has the big disadvantage in that it melts at about 26° C., and, as the optimum temperature for the multiplication of parasitic bacteria is usually 37° C., its function as a solid medium is considerably impaired. The proportion of gelatin employed is generally 10–15 per cent. by weight; this is added to broth. In hot climates the amount should be increased to 20–25 per cent. High temperatures interfere with its power of solidifying and care is therefore required in sterilization; steaming on successive days is the usual procedure. Gelatin, if too concentrated, tends to split when inoculated.

Agar or Agar-Agar is prepared from dried seaweed collected around the shores of China and Japan. It is added to broth to give a concentration of 2 per cent., and this constitutes an excellent solid medium, which is known as nutrient agar or simply agar. After the addition of the agar the broth is placed in the steamer for 1 hour to effect solution. The medium may be cleared by the addition of egg-white at a temperature of 55° C.; the egg-albumen coagulates and removes gross particles, which are easily held back on filtering through paper.

Nutrient agar, or agar, liquefies only on heating to 98° C. and sets at 45° C.; it is not damaged by temperatures of the autoclave and is thus an extremely satisfactory and serviceable medium. It is employed in the form of a slope, deep culture or a plate (Fig. 5).

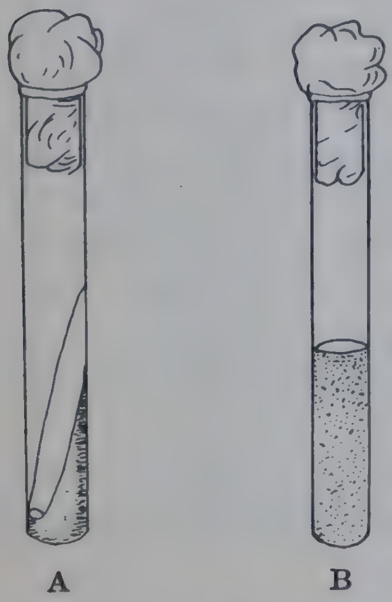


FIG. 5.—A, Agar slope. B, Deep agar for shake or stab culture.

Enriched Media. Simple media, such as broth or agar, are sometimes unsuitable for the isolation and subsequent growth of certain parasitic bacteria, such as the gonococcus, the pneumococcus and the influenza bacillus. It is consequently necessary to enrich them by the addition of appropriate substances; those usually employed are serum and ascitic fluid (10 per cent.), blood (5–10 per cent.) and glucose (1–2 per cent.). The resulting media, *e.g.*, serum-, blood-, ascitic fluid-, glucose-broth or agar, are termed "enriched media." The final dilution of the enriching substance usually varies from 1–10 per cent. for ordinary purposes.

The enrichment of broth is easily carried out by means of a sterile pipette. In the case of agar it is particularly important that the melted agar should be cooled to a temperature of 50°–55° C. before the addition of the enriching substances. Above this temperature the animal proteins tend to become coagulated and below it the risk of premature setting arises.

Selective Media. Media which favour the growth of certain bacteria are termed "selective" media. Chocolate agar, which is blood-agar heated to 98° C., favours the growth of the influenza bacillus, by converting the hæmoglobin into hæmatin and also by liberating the contents of the corpuscles. The contents of eggs mixed with 10 per cent. by volume of water, inspissated or coagulated by heating at 70° C., form a useful medium for the growth of tubercle bacilli. This is termed *Dorset's egg* medium, and its value may be enhanced by the addition of glycerine. Inspissated serum favours the growth of the diphtheria bacilli and is used extensively for the isolation of this organism as Loeffler's serum.

Selective media are also prepared by the addition of small amounts of inhibitory substances, which have a definite selective action on different bacteria; some are consequently inhibited while others grow luxuriantly. They are of great value in isolating an organism from material, such as fæces, which normally contains a large number of different bacteria. Important examples are the desoxycholate-citrate, tellurite, Wilson and Blair, and Lowenstein-Jensen media which have proved invaluable for the isolation of dysentery, diphtheria, typhoid and tubercle bacilli respectively.

In media containing carbohydrates substances which show a different colour in an acid and alkali substrate can be added; these substances are termed *indicators*. Those commonly used are litmus, phenol-red and Andrade's solution, which is prepared by decolorizing 0·5 per cent. aqueous acid fuchsin with caustic soda.

Sterilization. All media used for the cultivation of bacteria must obviously be free from contaminants and therefore must be sterilized before use. Several different methods are available according to the nature of the medium. Heat in the autoclave at 105°–120° C. is usually employed, but this reduces the value of the medium in some cases, *e.g.*, gelatin, sugar solutions and certain media containing serum, blood or ascitic fluid; heat at lower temperatures on successive days or filtration through bacterial filters is then necessary. This question is discussed in more detail later (p. 54).

The Use of Culture Media

In order to derive the maximum information from the cultivation of bacteria it is essential that the cultures should be prepared in a satisfactory manner. Under ordinary circumstances the procedure varies with the type of medium.

Fluid media are prepared either in ordinary test-tubes (6 × ½ in.) containing 5–10 c.c., small screw-cap bottles or flasks of varying sizes (50–250 c.c.). The flasks and test-tubes are both stoppered with plugs of cotton-wool.

Solid media are prepared either in test-tubes, small screw-cap bottles or Petri dishes. The medium in the test-tube is either upright or sloped. An ordinary upright tube contains about 10 c.c. The form most commonly employed is a sloped tube or *slope.* About 5 c.c. of melted agar are placed in a test-tube and allowed to cool in a slanted position. In this way a large surface area is obtained from a relatively small quantity of medium.

When a large and readily accessible surface is required, about 10 c.c. of the melted medium are allowed to cool to 5°–10° C. above the setting temperature and are then poured into a Petri dish. Condensation of steam on to the medium or the lid is avoided by allowing the medium to set at 37° C. with the lid not completely covering the medium but resting on one side of the dish. Plates are particularly useful in the isolation of pure cultures from grossly contaminated material.

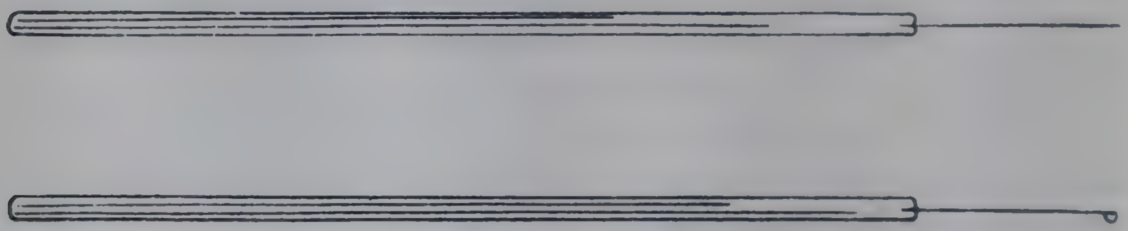


Fig. 6.—Platinum loop and needle.

The Inoculation of Media. The instruments commonly used to inoculate or seed a medium are the platinum loop and needle. The platinum loop consists of a piece of platinum wire, 3 in. long, one end of which is fixed into an aluminium holder while the free end is bent in the form of a loop. The loop must be closed and quite flat. The needle is similar, but without the loop (Fig. 6). Owing to the high cost of platinum, resistance wire is usually used for class purposes. This cools slower than platinum and it is important after flaming to allow the wire adequate time for cooling before use.

Fluid material, such as serum, is sometimes added to a medium by means of capillary or Pasteur pipettes; the latter are made by heating glass tubing and drawing out the melted glass to form two pipettes.

The inoculation or seeding of a medium requires much practice before a satisfactory technique is acquired. The method varies with the medium used. When the medium is contained in a test-tube, this should be held at its lower end in the left hand between the thumb and the first two fingers. The platinum loop is held in the right hand and is sterilized in a bunsen flame, while the plug of the tube is removed by the little finger and the palm of the same hand; the mouth of the tube is passed through a bunsen flame and the inoculation carried out, after which the plug is replaced. By the use of such technique it is possible to observe all the stages of the procedure. A common fault is to grip the middle of the

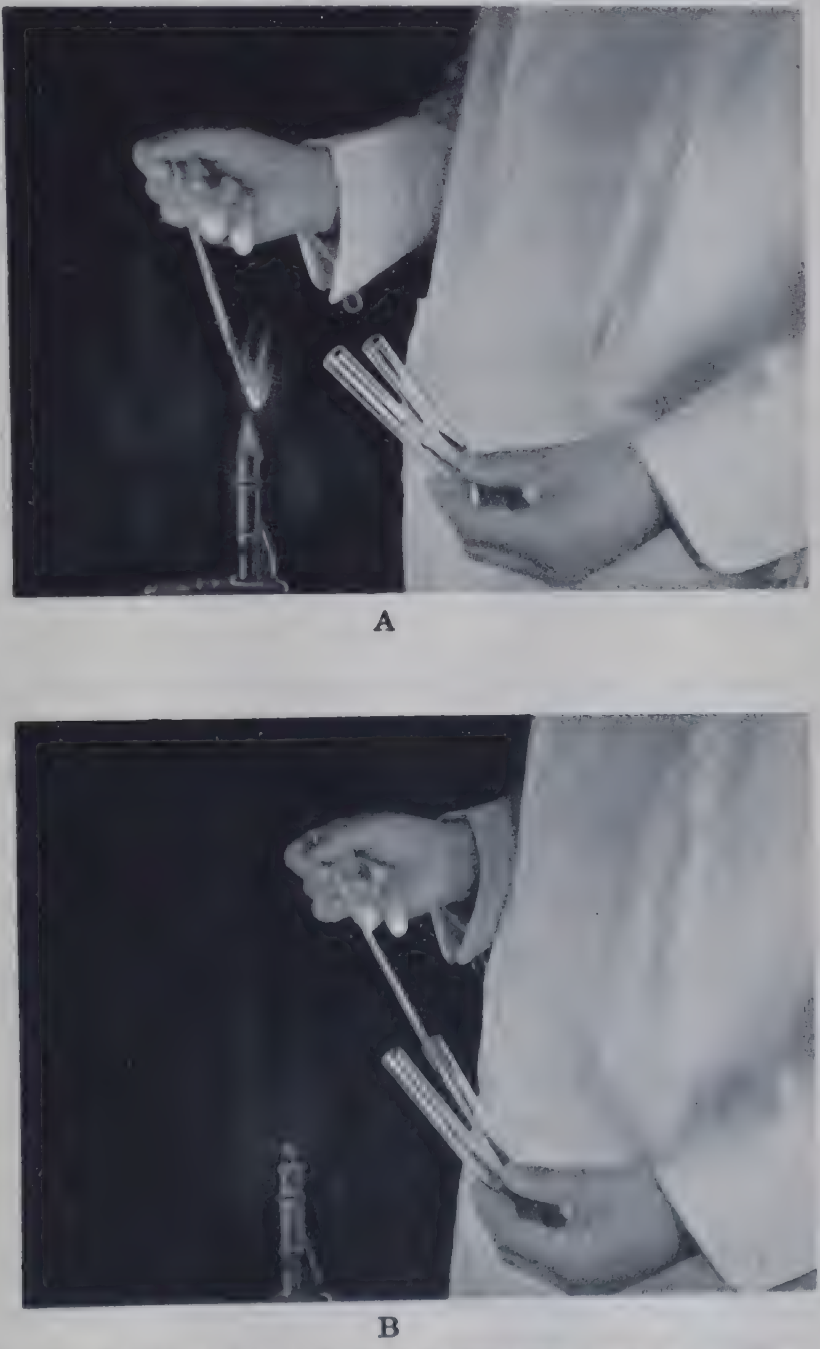


Fig. 7.—Method of subculturing from one slope to another.

tube with one hand and then to plunge the loop blindly inside. In inoculating from one tube to another, both tubes should be held between the thumb and the first two fingers of the left hand. One plug is removed by pressure applied by the little finger and the palm, and the second between the little and ring fingers of the right hand (Fig. 7).

The plugs must not be removed and placed on the bench during the seeding of the tube. Such a practice is certain to result in contamination.

In seeding fluid media the charged loop should be passed immediately below the surface and rubbed gently against the side of the tube. Vigorous agitation of the loop should be avoided.

In seeding solid media the arrangement of the medium must be considered. For a slope culture the loop should be pressed gently but firmly on the surface of the lowest part of the medium and drawn up the surface by passing from side to side. In this way the inoculum is thinned as a result of the streaking. Upright media may be used either as stab or shake cultures. In a *stab* culture the charged needle is passed vertically down the centre of the medium. In a *shake* culture the medium is melted and allowed to cool to a few degrees above the setting temperature, at which stage the inoculation is made either by a loop or a capillary pipette. The tube is then rotated between the hands, to distribute the inoculum, and allowed to cool.

The platinum loop or needle must always be flamed before and after use ; after the initial heating it must naturally be cooled to a temperature not injurious to the bacteria. This is a detail often forgotten by the student and may be responsible for the failure of organisms to grow in culture.

A medium which has been successfully inoculated is termed a *culture*. When only one species of bacterium is present it is termed a *pure* culture. The inoculation of a second medium from a previous culture is termed a *subculture*. It is important that all cultures should be carefully labelled and the source of the material and date clearly indicated.

The Incubation of Cultures. Inoculated media are placed in a thermostat at a temperature suitable for their growth. Thermostats or incubators working at 37° C. are necessary for the study of the parasitic bacteria. The incubators, water-jacketed and either gas or electrically heated, are regulated by capsules. In large laboratories heat-regulated rooms are sometimes available. Agar, but not gelatin, plates are incubated in an inverted position :

gelatin media are usually placed in a special incubator at a temperature of 22° C.

Anaerobic Methods. The atmospheric conditions of cultivation also require attention. The anaerobic bacteria will not grow in the presence of free oxygen in the ordinary media, while microaerophiles require a greatly reduced O_2 tension. It is therefore necessary to remove or exclude the atmospheric O_2 in order to obtain multiplication of these bacteria. The following methods may be used for this purpose :—

(1) Oxygen can be excluded from the medium where growth is taking place. Test-tubes containing a relatively large amount, 10–15 c.c., of the medium, solid or fluid, are used. In the case of the solid media the culture may be either a shake or a stab ; in both growth appears some distance below the surface. With fluid media growth frequently occurs at the bottom of the tube. As all media contain a certain amount of residual free O_2, this should be removed by boiling the medium before use. The efficacy of this method is increased by the addition of some reducing substance to the medium. The reducing agents commonly employed are glucose and pieces of sterile tissue, such as muscle or kidney. Anaerobiosis is also assisted by layering the surface of the medium with sterile vaseline or paraffin. Cultures, so prepared, are incubated in the ordinary way.

(2) Oxygen may also be removed from the atmosphere by means of pyrogallic acid or by the growth of other bacteria ; the latter procedure is not, however, employed as a routine measure. The simplest method of using pyrogallol is to put this and caustic soda or potash, 25 per cent. strength, on the top of the plug of an ordinary culture, either in broth or on a slope, and cover with melted paraffin. The combination of the alkali and pyrogallic acid readily absorbs oxygen and reasonably satisfactory anaerobic conditions are obtained inside the tube ; surface growth can be obtained.

(3) The most satisfactory method of cultivating the anaerobes is by incubating cultures in a jar, from which the oxygen has been removed and replaced by hydrogen. The best apparatus for this purpose is the McIntosh and Fildes' jar or some modification. The McIntosh and Fildes' jar is made of durable glass and has a tight-fitting lid that can be firmly clamped down. On the undersurface of the lid there is a piece of palladinized asbestos surrounded by resistance wire, which is connected to two terminals, the heating element being protected by wire gauze. The lid is also pierced

by a tap. Hydrogen is added slowly after removal of the air and a current passed through the resistance wire surrounding the palladinized asbestos, which is consequently heated and acts as a catalyst. Combination of the O_2 and H_2 takes place and water forms. The consumed oxygen is gradually replaced by hydrogen until finally all the oxygen is used up. In order to indicate the presence of anaerobiosis a weak aqueous solution of methylene-blue is added; this in the absence of oxygen becomes colourless.

All types of cultures, *e.g.*, plates and tubes, may be grown under such conditions.

A useful modification of the McIntosh and Fildes' jar is that by Wilson. This jar is constructed of a copper alloy, and consequently can stand much greater strains than the glass jar. Air is first removed by means of a vacuum pump and hydrogen is then slowly added; an electric current is then passed through. The main disadvantage of this particular jar is that the contents are not visible and so the behaviour of the cultures cannot be studied without the inconvenience of opening the jar and removing the tubes and plates. Hydrogen may be obtained from either a Kipp apparatus or a cylinder, and should be added slowly.

(4) A further method is the addition to a fluid medium of active reducing agents which permit the growth of anærobes in the presence of air by removing free oxygen. Satisfactory substances are iron, sodium thioglycollate and vitamin C (cf. p. 346).

The Isolation of Pure Cultures

In order to study the cultural characteristics of a bacterium it is essential to obtain a pure culture. It frequently happens that material sent for examination contains a number of different bacteria, and the first stage of the examination is obviously the isolation of the individual species. Since the introduction of solid media by Koch in 1881 considerable progress has been made in this direction. Numerous methods have been described, and while some are of doubtful value others have proved highly satisfactory. The important methods will be briefly considered.

1. By Dilution. Before the introduction of solid media the isolation of pure cultures was a matter of considerable difficulty. Material to be examined was diluted with some suitable diluent, such as water, saline or broth, and seeded into fluid media. This method is obviously unreliable as it only produces a pure culture of the organism present in the greatest numbers. It is largely used at the present time in conjunction with the plating method.

2. Plating. Cultures are prepared on solid media in Petri dishes by streaking. The platinum loop is charged with material and a series of strokes made on the surface of the medium. The most satisfactory method of thinning the bacterial growth is shown in Fig. 8. The first strokes are made at the side 1, the plate is rotated and further streaks made, starting at position 2, the plate is again rotated to 3 and a final series of strokes made. It is important that the surface of the medium should be dry, otherwise growth tends to be confluent.

A spreader, made by bending a piece of heated glass rod, can be used instead of the loop, but the results are not so satisfactory when many bacteria are present in the material under examination. When, however, it is suspected that the material contains a large number of organisms, it is advisable either to inoculate several plates from one drop or to make serial dilutions which can be cultivated separately. Subcultures are made from the separate colonies (Fig. 9).

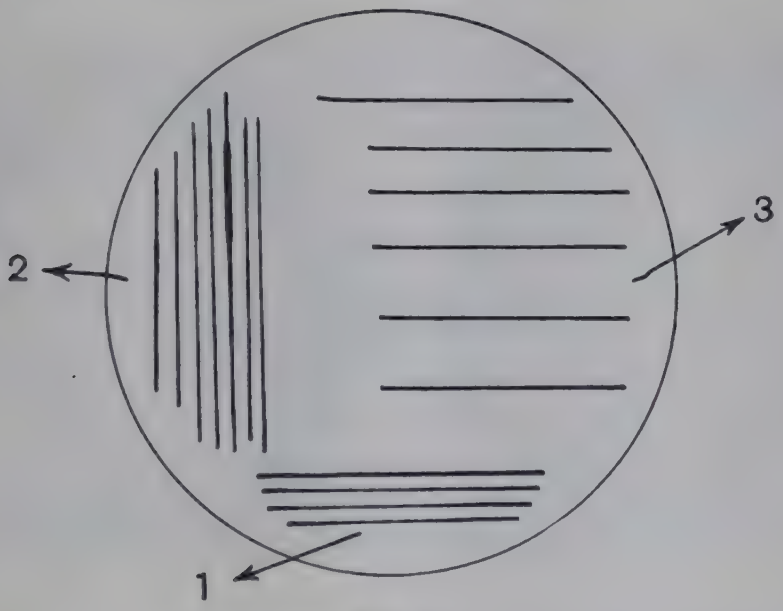


Fig. 8.

3. Optimum Temperature. It is sometimes possible to obtain pure cultures by incubation at different temperatures. The optimum temperature for growth varies with different organisms; parasitic bacteria grow best at 37° C., while the saprophytic forms usually grow luxuriously at 20°–25° C. The thermophilic bacteria may be isolated by incubation at 60° C., at which temperature most bacteria are unable to multiply.

4. Aerobic and Anaerobic Cultivation. Bacteria vary in their reaction to atmospheric conditions. Many will grow under both aerobic and anaerobic conditions, but others are either strict aerobes or anaerobes. Thus by cultivating in the presence of, and in the absence of, oxygen it is possible to separate the obligate aerobes from the obligate anaerobes.

5. Selective Media. One of the most frequently applied methods of obtaining pure cultures is by the use of selective media, which usually contain inhibitory substances and indicators. The use of Wilson and Blair, tetrathionate broth and selenite media for the isolation of the typhoid-paratyphoid bacilli from fæces has yielded excellent results. Potassium tellurite is added to serum or blood agar to form an excellent medium for the isolation of the diphtheria bacilli, which produce blackish colonies. Dysentery bacilli can now be readily isolated from the fæces of convalescent cases and apparently healthy carriers by means of the recently introduced desoxycholate-citrate medium. The presence or absence of hæmolysis on blood agar plates is also a useful criterion for identification, especially in the case of the streptococci. The addition of litmus or phenol-red to medium containing carbohydrates is sometimes useful; bacteria fermenting the carbohydrate form a reddish growth with litmus and a yellow growth with phenol-red.

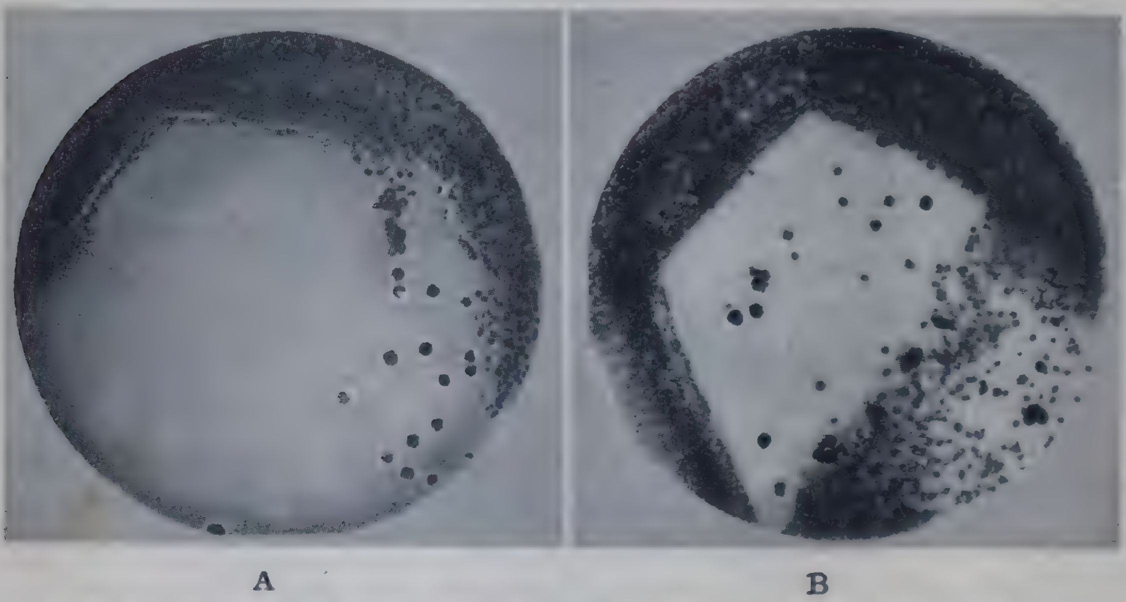

A B

Fig. 9.—Surface of plate with discrete colonies obtained from heavy suspensions of (A) a pure culture, and (B) a mixed culture. (The two distinct types of colony are clearly shown.)

6. Heating. Spore-bearing bacteria are more resistant to heat than the vegetative forms. By heating a suspension at 80° C. for 10 minutes vegetative bacteria are destroyed while the spores are unaffected. On subsequent cultivation the spores germinate into vegetative forms. This method is used largely for the isolation of anaerobic spore-bearing organisms from contaminated material.

7. Animal Inoculation. In some cases the most satisfactory method of isolating bacteria is by the inoculation of susceptible laboratory animals, such as mice or guinea-pigs. This method is used extensively for the isolation of the tubercle bacillus from milk, pus, etc.; it is also employed for isolation of the pneumococcus from sputum in cases of pneumonia, and the anthrax bacillus from contaminated material, such as shaving-brushes.

The Identification of Bacteria

The identity of an organism can seldom be established solely on the results of a morphological examination, and it is usually necessary not only to prepare cultures but also to carry out a number of biochemical tests. In some cases even these examinations are insufficient, and serological and/or pathogenicity tests are then required. It is, however, usually possible to obtain much valuable information of the identity of an organism from an examination of its cultural reactions. It is important to note that the behaviour of any particular bacterium is by no means constant, but is dependent on several factors, particularly the environmental conditions and age of the culture. Under favourable conditions, however, the results are sufficiently constant to be useful for the purposes of identification. A series of cultures in different media is usually prepared; an indication of those likely to yield useful results is frequently given by the morphological and staining reactions of the bacterium. Pure cultures are obviously essential, and it is necessary to examine them daily for varying periods, in some cases up to several weeks, *e.g.*, cultures for tubercle bacilli.

The various types of media furnish different information; they may be conveniently grouped into three types: (1) fluid, (2) solid, and (3) those used for biochemical tests.

Fluid Media. In examining cultures in the ordinary fluid media, such as broth, attention is directed mainly to three points:—

1. The presence or absence of a surface scum or "pellicle."
2. The appearance and degree of turbidity in the fluid.
3. The presence of a deposit and its nature, *i.e.*, granular, tenacious, heavy, light, readily dispersed, etc.; the character of a deposit is satisfactorily determined by rotating the tube between the hands.

Solid Media. The examination of the growth on or in a solid medium is largely one concerned with the characters of the individual colonies. Discrete colonies are considered to represent the growth of one particular bacterium or an aggregation of the same bacterium, as with the staphylococcus. The points about the colonies to be noted are: size, shape, edge, regularity of structure and surface, opacity, consistency, pigmentation and the readiness with which they can be removed from the medium. Many of these details are detected by observation through a hand-lens or the low powers of the microscope. In a deep or shake culture the distance of the growth from the surface of the medium should be noted.

Biochemical Activities. The biochemical tests often utilized to identify bacteria are those concerned with the fermentation of carbohydrates and the breaking down of proteins. The capacity of organisms to split fats, to reduce nitrates and certain dyes, such as methylene-blue, to produce catalase and to form NH_3 and H_2S may also be applied in diagnosis. The fermentation of carbohydrates or saccharolytic activity is usually tested by growing the bacteria in peptone water containing 0·5–1 per cent. of the various carbohydrates. Many bacteria in breaking down the sugar molecule produce both acid and gas, while others produce only acid and no gas. The production of gas is detected by adding to the medium a narrow inverted tube, at the top of which some of the gas collects. This arrangement is known as a Durham tube. Acid formation is detected by the addition of an indicator, such as litmus or Andrade's solution. Litmus turns red in an acid medium, while the neutral point of Andrade's solution is pH 7·2, when it is faintly yellow or colourless; in the presence of acid it becomes red.

Some parasitic bacteria, *e.g.*, pneumococcus and meningococcus, do not grow in the peptone-sugar solutions and consequently richer media are required. The one commonly used is Hiss's serum water, which is serum diluted with two or three times its volume of distilled water plus the carbohydrate and an indicator. Acid formation is indicated by a change of colour and coagulation of the medium. The carbohydrates or "sugars" in common use are lactose, maltose, mannite, saccharose, dulcite, glucose and inulin.

The breaking down of proteins or the proteolytic activity of an organism is tested in various ways: the liquefaction of gelatin, serum and egg-media; the peptonization of milk; the digestion and blackening of meat with the production of an offensive odour; and the breaking down of peptone to form either indole or ammonia.

Test for Indole. The production of indole may be detected by two methods. One is to insert between the plug and the wall of the tube a sterile strip of filter paper saturated with oxalic acid ; on the production of indole from the peptone water, which must contain adequate quantities of tryptophane and be free from sugar, the paper turns pinkish. The other method is to incubate cultures in broth or peptone water for 2–5 days, then add a few drops of a 2 per cent. solution of paradimethylaminobenzaldehyde in absolute alcohol ; if indole is present the medium turns pinkish, the reaction is generally hastened by the addition of a saturated solution of potassium persulphate. This test is styled by different workers Ehrlich's rosindol reaction or Bohme's reaction.

Other tests depending on the metabolic activity of bacteria are the Voges-Proskauer and methyl-red reactions, which are used extensively in the bacteriological examination of water.

Voges-Proskauer Reaction. This reaction is determined by adding strong KOH (15–40 per cent.) to a 2–4 day glucose-broth culture ; when positive a pinkish colour appears slowly and may take several hours to develop fully. This colour formation is due to the production from glucose of acetyl-methyl-carbinol, which, in the presence of a strong alkali and atmospheric oxygen, is oxidized to diacetyl, and this reacts with the peptone of the broth to give the pinkish colour. The sensitivity of the reaction is considerably increased by the addition of small amounts of either creatine or α-naphthol (5 per cent.) alcoholic solution immediately before adding the KOH solution.

Methyl-Red Reaction is merely a test for the production of acid. After growth in a glucose-broth medium a few drops of a methyl-red solution (0·1 gm. methyl-red dissolved in 300 c.c. alcohol made up to 500 c.c. with distilled water) are added to the culture ; the appearance of a red colour indicates a high H ion concentration and is a positive reaction.

CHAPTER V

THE MULTIPLICATION OF BACTERIA

The factors involved in the reproduction of bacteria are both numerous and complex. The complex molecules provided by the medium in which the bacteria are growing are broken down and from the resulting simpler substances the bacterial protoplasm is synthesized. In spite of the different processes concerned, the multiplication of bacteria under favourable conditions tends to take place in a regular manner, and, after seeding a fluid medium with a bacterium, the various stages of growth can be determined by counting the bacteria, living and dead, at regular intervals of time. By such means it is possible to examine the effect of various factors on the rate and degree of bacterial growth *in vitro*, *i.e.*, in the test-tube. A knowledge of the factors influencing bacterial multiplication *in vitro* is valuable in that it throws some light on the development of bacteria *in vivo*.

Before discussing the question of bacterial growth, a brief consideration of the methods of counting bacteria is necessary. The two principal aims in counting bacteria are to estimate :—

(1) the number of living bacteria, *i.e.*, a viable count.

(2) the number of living and dead bacteria, *i.e.*, a total count.

1. Viable Count. The number of living bacteria in a suspension is determined by plating a standard amount of serial decimal dilutions of the suspension. Dilutions in saline up to $1/10^7$ or $1/10^8$ of the original suspension are made ; a fresh pipette must be used for each bacterial transfer. Half to 1 c.c. of each dilution is placed in a Petri dish, melted agar, cooled to 50°–55° C., is added and the contents of the plate mixed by gentle agitation. After incubation at the optimum temperature for 24–48 hours, the total number of colonies on plates containing a countable number is obtained and an average made after the dilutions have been adjusted. In this manner the number of living organisms per c.c. can be estimated with a moderate degree of accuracy.

2. Total Count. The number of bacteria, both living and dead, may be estimated by various methods, but none is particularly accurate. The methods in frequent use are :—

(A) The direct count obtained by placing a drop of the bacterial suspension on a Helber slide which has a graduated scale, similar to a Thoma-Zeiss hæmocytometer slide and a special cover-slip.

(B) By comparing the opacity of the suspension with a prepared series of opacity tubes made with barium sulphate. Standard opacity tubes with a regulated scale of counts for various bacteria are available ; they are prepared by Messrs. Burroughs, Wellcome and Co.

(C) Wright's method consists in mixing equal amounts of blood and a known dilution of the bacterial suspension, making a film, staining and counting the number of bacteria and R.B.C. in the same given number of fields. Taking the R.B.C. count to be 5,000,000 per c.mm., the number of bacteria can be determined by simple proportion.

By means of repeated counts after seeding bacteria in fluid media it has been found that bacterial multiplication can be divided arbitrarily into four phases—(*a*) lag, (*b*) logarithmic, (*c*) stationary and (*d*) decline.

(*a*) During the first or *lag phase* there is no appreciable growth of the bacteria. The duration of this phase is subject to considerable variation, usually from $\frac{1}{2}$ to 8 hours. Not infrequently there is an initial fall in the count due to the death of some of the inoculated bacteria. The true explanation of this phase is somewhat controversial, but it doubtless represents an adjustment of the bacterium to the change of environment.

(*b*) The second phase is the stage of most rapid multiplication, and is termed the *logarithmic phase*. Increase tends to occur by geometric progression as each bacterium divides into two daughter-cells ; this statement is, however, not strictly correct, because some of the bacteria die during this stage. It has been estimated that the generation time of the typhoid bacillus may be as brief as 20 minutes during the logarithmic stage.

(*c*) The second stage is followed by a period at which the viable count remains steady ; this is consequently termed the *stationary phase*. During this period the number of bacteria dying balances the formation of fresh forms. The reason for the marked decrease in the multiplica-

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tion of the bacteria has not been determined with certainty, but it appears to depend to some extent on the production of noxious substances during the second phase and to the lack of oxygen in the case of some aerobic organisms.

(*d*) The final stage is one of decline, the number of viable forms gradually decreases until all are dead. This may take several months or only a few days and depends largely on the production in the medium of substances injurious to the bacteria. This is illustrated by the different curves obtained by growing an organism which ferments glucose, *Bact. aertrycke*, in peptone water and peptone water plus 2 per cent. glucose (Fig. 10).

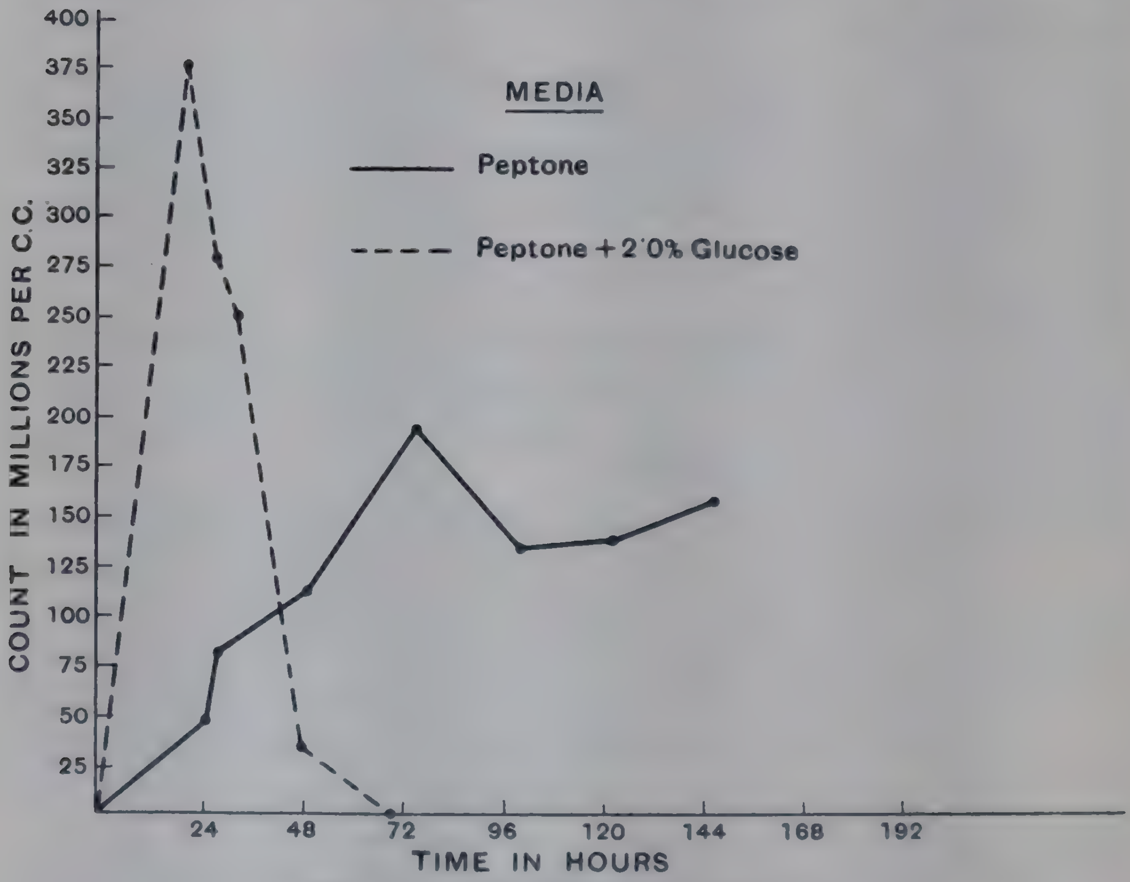


FIG. 10. (After Heap and Cadness.)

The peptone culture shows a relatively slow logarithmic phase and prolonged stationary and decline phases, whereas when glucose is added to the medium there is a rapid increase in the viable count and a correspondingly rapid decline. The explanation is that in the latter culture the glucose is fermented with the production of acid, which kills the bacteria and so considerably shortens the decline phase.

The growth curve of the various bacteria is influenced by a number of factors, the most important of which are the temperature of incubation, the nature, age and amount of the seeded bacterium and the medium employed. The use of the optimum temperature, a satisfactory medium and a fresh actively growing organism considerably shortens both the lag phase and the generation time, while under unfavourable conditions both these phases may be greatly prolonged.

Dormancy. A phenomenon related to growth is that of dormancy or suspended animation. At temperatures lower than those favourable for growth many bacteria do not multiply, but are able to remain alive for considerable periods. Use is made of this fact in the preservation of stock cultures, many of which are kept at 0° C. In certain cases on seeding spores or bacteria into a suitable medium and incubating at a favourable temperature growth does not take place for some time. This has been noted to occur not infrequently with the spores of *Cl. botulinum*, which under favourable conditions may not germinate for many days. This fact has been held responsible for the difficulty sometimes encountered in sterilizing by the intermittent method material contaminated with this organism.

Symbiosis and Antagonism. In nature organisms are seldom found alone, many varied species usually multiply side by side. Substances such as acids which are injurious for certain species may be produced and so, in certain cases, a preponderance of special types may occur. It has been noted on occasions that, when two organisms are growing together under artificial conditions, the growth of one may have a beneficial effect on the growth of the other ; this is sometimes termed *symbiosis*. An example is the influence of the staphylococcus on the growth of the influenza bacillus on blood agar media due to the production by the former of the V accessory factor.

In other cases where two organisms are growing together it is found that one may have a definitely harmful effect on the other. This is termed *antagonism*. An illustration of this is the inhibition of the growth of the gonococcus by *Ps. pyocyanea* and the interference by staphylococci with the multiplication of the diphtheria bacilli. This antagonism may be due to a struggle for food between the two bacteria or to the production in the medium by one organism of substances injurious to the other.

CHAPTER VI

THE DESTRUCTION OF BACTERIA

The initial experiments of Lister and his subsequent introduction of antiseptic surgery directed attention to the practical importance of bacterial destruction. An enormous quantity of work has since been carried out on this subject, and the practical applications of the results are now demonstrated in many different spheres, such as hygiene, surgery, the canning of foods, refrigeration and meat preservation.

The subject of bacterial destruction is one of great complexity, due mainly to the large number of variable factors involved in the process. The following points require close attention in the examination of any disinfection process : (*a*) the nature of the organism involved—spore-bearers are more resistant than vegetative organisms, among which great individual variation in susceptibility to various agents is found ; (*b*) the strain of the organism and the age of the culture are important, *e.g.*, the smooth-rough variation is accompanied by changes in resistance ; (*c*) the temperature of the test—the higher the temperature the more rapid the action ; (*d*) the time of the test and the number of organisms present—the longer the time of action the more organisms destroyed ; (*e*) the menstruum in which the reaction is taking place—the action of many bactericidal agents is greatly impaired by the presence of extraneous organic matter, such as protein, but tends to be increased by an acid reaction ; (*f*) the concentration of the disinfectant—the greater the concentration the more rapid the action, this is not, however, a simple relationship.

It is important to realize that methods readily applied in the laboratory for destroying bacteria can seldom be used for this purpose in the tissues, as agents that kill bacteria are usually equally destructive to the body-cells. Bacteriologists and chemists have made repeated efforts to find substances which are harmless to the tissues and yet are injurious to bacteria, but without any marked success until the recent introduction of the sulphonamides and penicillin as therapeutic agents. The value of the former substances is, however, due to bacteriostatic rather than bactericidal activity resulting from interference with bacterial metabolism.

The most important application of bactericidal agents in medicine is in the limitation of infection by the destruction of bacteria outside the body, as in the treatment of infected material such as fæces, urine, instruments, clothes, sewage, sputum, etc.

Agents that destroy bacteria are termed *disinfectants* or bactericidal agents ; while those that do not exercise a bactericidal action but only inhibit bacterial growth are termed *antiseptics* or *bacteriostatics*. On removal to more favourable surroundings of bacteria treated with antiseptics, growth and reproduction again take place. Disinfectants when suitably diluted usually have a bacteriostatic action. These terms are often used in a loose sense and have lost much of their original significance.

The process of bacterial destruction varies with the different bactericidal agents. The usual action of the agents is to coagulate the bacterial protoplasm, as in the case of heat and the metallic salts. In other cases the protoplasm is oxidized and burnt up, as with the halogen group ; in some instances the destructive action is secondary to a physical interference with bacterial metabolism, the agent acts on the surface of the cell and so prevents its intake of nutriment.

Agents destructive to bacteria are divided into two main groups—physical and chemical.

Physical Agents

Physical agents with a bactericidal action are light, desiccation, electricity and heat ; of these, heat is the one of greatest practical importance.

Light. It has long been recognized that light exerts a bactericidal action and that in this respect direct sunlight is more effective than diffuse daylight. Sunlight not only destroys bacteria, but it may also alter a culture medium so that it becomes unsuitable for bacterial multiplication: The bactericidal action of the sun can be readily demonstrated by seeding the surface of two plates with the same bacterium and exposing one to the direct sunlight. On subsequent incubation it will be found that the growth on the unexposed plate is much more profuse than on that exposed. The lethal power of light is due to the activity of the short waves. An examination of the spectrum shows that the long waves, *i.e.*, infra-red and red rays, have very little bactericidal action, while the ultra-violet rays are those mainly responsible for the destruction of bacteria. Moreover, this destructive action is not restricted to bacteria, but is exercised on all unicellular organisms and also the body-cells. The power of penetration of these rays is, however, low, so that the action is limited mainly to the surface growth. The mode of action is not definitely known, but the indications are that it is more a chemical than a physical process. No evidence has been obtained to suggest that the destructive action is due to any actual increase in temperature resulting from the application of the rays. Ultra-violet rays are not commonly employed for bacterial destruction, their main application has been in the treatment of drinking and swimming-bath water.

It has recently been found that the lethal action of certain rays may be considerably increased by the addition of some fluorescent dye, such as eosin or methylene-blue, to the bacterial suspension. Rays, ordinarily inert, become definitely bactericidal under such conditions. This phenomenon is termed "photo-dynamic activity". The factors involved are not fully understood, but the action is probably due to an activation of oxygen or some oxidation product by the process.

Conflicting evidence has been obtained regarding the action of X-rays and radium, but it would appear that neither has any marked bactericidal action.

Electricity. The use of electric currents as a means of sterilizing fluids, such as milk, has been tried, but the results have not been satisfactory. Some bactericidal action has been observed, but it is probable that this depends upon the associated production in the medium of heat and bactericidal substances, such as H_2O_2 and acids, rather than to any direct action of the electric current itself.

Desiccation. Deprivation of water may have a deleterious action on bacteria. The survival time of bacteria after drying depends on a number of varied conditions, including the bacterial species, the menstruum, the temperature and method of drying. Spores offer much more resistance than vegetative organisms. It has recently been found that bacteria when frozen and dried *in vacuo* may conserve their viability and virulence for considerable periods of time ; this procedure is now used, in some instances, as a means of preserving stock cultures. Many of the earlier views on the bactericidal action of desiccation appear to require revision.

Heat. Heat is the most widely applied bactericidal agent available. It has already been noted that, on increasing the temperature above that required for optimal growth, reproduction gradually decreases while the destructive action becomes more and more marked. This destructive action continues until all the bacteria in the suspension are destroyed.

The destructive action of heat can be utilized in two forms—dry heat and moist heat.

Dry Heat. Dry heat, as a general rule, is not such a powerful bactericidal agent as moist heat, owing to its relatively low power of penetration. Destruction takes place as a result of coagulation of the cell protein, and vegetative bacteria are more readily destroyed than spores. Practically all vegetative forms are killed by 1½–2 hours' exposure at 100° C.; spores, however, may require a temperature of at least 140° C. acting for 3 hours or of 160° C. for 1 hour. The main applications of dry heat are :—

(1) Incineration for the destruction of carcases or contaminated material ;

(2) The naked flame for sterilizing platinum loops, needles or other non-inflammable substances.

(3) Hot air oven, 160° C. for ½–1 hour, for sterilizing glassware, such as Petri dishes, pipettes and flasks.

Moist Heat. The marked bactericidal action of moist heat was demonstrated and applied by Koch. Much lower temperatures are required to destroy both vegetative and spore-forms than with dry heat. Boiling water readily destroys vegetative organisms, but certain spores may resist its action for 24 hours or longer. For this reason steam under pressure, at temperatures of 115°–130° C., is usually employed for sterilization. Steam at 120° C. (*i.e.*, under pressure of 15 lb.) acting for ½ hour is sufficient to destroy practically all spores.

The superior action of moist heat is due to several reasons :—

(*a*) its greatly increased powers of penetration ; it has been found that the temperature inside a bundle of flannel, subjected to dry heat at 140° C. for 4 hours, was only 83° C. After 1½ hours' treatment with moist heat at 120° C. the internal temperature was 117° C. ;

(*b*) protein is more easily coagulable by heat when it contains abundant water. Moist heat tends to increase the amount of moisture present in the bacterial protein, whereas dry heat will dehydrate it and so raise the temperature necessary for coagulation ;

(*c*) steam is a better conductor of heat than air.

It is important to note that the addition of even a small amount of acid or alkali to the medium in which the bacteria are suspended greatly enhances the efficacy of moist heat.

The main uses of moist heat are given below :—

(1) The autoclave, in which steam is under regulated pressure, is an essential feature of a bacteriological laboratory. It is used for the sterilization of most media and for the destruction of old cultures. The steam must be saturated and not superheated, as in the latter case it acts more like hot air.

(2) The steamer, in which the steam is not under pressure. In the preparation of certain media, such as gelatin, care must be taken not to overheat, and sterilization is frequently carried out by steaming at 100° C. for ½ hour on 3 successive days ; this process is termed Tyndall's intermittent or fractional sterilization. The principle is that, after heating, the medium is incubated to allow the spores to germinate and so become more readily destroyed by the steaming process on the following day. The method obviously fails if the spores do not germinate during the period of incubation.

(3) The sterilization of instruments, scissors, knives, syringes, etc., is often carried out by boiling in a water-bath.

(4) Pasteurization of milk by heating at 62° C. for 30 minutes is used to kill pathogenic organisms, which are generally non-sporing forms, *e.g.*, tubercle bacilli and *Br. abortus*.

(5) Heat at 56°–60° C. is frequently employed to kill vegetative bacteria in the preparation of vaccines. While most vegetative bacteria are destroyed at this range of temperature, some, *e.g.*, *Strept. fæcalis*, may survive such treatment.

Chemical Agents

A considerable number of chemical agents exercise a definite bactericidal action, but unfortunately the majority, if not all, have also an injurious action on the body-cells. It thus follows that although the bactericidal action may be easily obtained in the test-tube, the therapeutic application of chemical agents is often impracticable ; at dilutions harmless to the tissues the bactericidal action is frequently lost. The researches of biochemists have introduced various synthetic products in the hope of overcoming this difficulty, but until the recent introduction of the sulphonamide group of drugs, the results had not been entirely satisfactory.

A description of all chemicals possessing bactericidal properties is impossible, but a consideration of the different classes into which these agents have been grouped is both useful and instructive. There is no uniform law governing their action ; many variable factors are involved, and each may modify considerably the efficiency of the destructive process. Factors of particular importance in this respect are the nature of the agent, the organism, temperature, time of action and the menstruum in which the reaction is taking place.

Distilled Water. There appears to be no universal opinion about the bactericidal action of distilled water. Some destructive action seems to take place, but in many experiments the presence of impurities in the water has not been satisfactorily excluded. The different species of bacteria vary considerably in their behaviour to distilled water ; spores, however, are definitely resistant to its action. Osmosis does not appear to play an important *rôle* in whatever destructive action distilled water may possess.

Acids. The bactericidal action of acids is largely dependent on the degree of electrolytic dissociation exhibited, *i.e.*, on the concentration of the H ions. Inorganic acids, as a general rule, are more active than organic acids as they dissociate more readily. Hydrochloric acid and sulphuric acid are most commonly used in practice ; excess is necessary as they tend to combine easily.

Alkalies. The disinfectant effect of alkalies also tends to be proportional to their electrolytic dissociation, *i.e.*, to the concentration of the OH ions ; KOH is one of the most active as it readily dissociates. In some cases, where the base is an alkaline earth, as barium hydroxide, the cation also has a toxic action on the bacteria. As a general rule H ions are more effective than OH ions. Tubercle bacilli are more resistant to alkalies than other vegetative bacteria. The disinfectant action of soaps is due mainly to the presence of alkalies.

Oxidizing Agents. Substances which readily give up oxygen are usually active disinfectants. They are consequently widely employed in practice, particularly hydrogen peroxide and potassium permanganate.

Halogens. Members of the halogen group are strong disinfectants, but, as they break down rapidly in aqueous solution and also damage organic matter, they are difficult to apply in practice. Their activity tends to be inversely proportional to their atomic weights, the order of toxicity is therefore chlorine, bromine and iodine.

Chlorine is used extensively as a disinfectant. It is frequently added to drinking and swimming-bath water in a free state. It is also employed largely in compound form as chloride of lime, chloramine, eusol and Dakin's solution, which is bleaching powder treated with washing soda. Iodine is widely applied as the tincture or in a dry form as iodoform ; tincture of iodine is one of the most satisfactory disinfectants for sterilizing the skin.

Alcohols and Ether. Absolute alcohol has only a slight bactericidal action, but when diluted to 50 per cent. strength it has marked disinfectant properties. Ether is not very active. A product of methyl alcohol, formaldehyde, is particularly efficacious and has a wide application as formalin, which is a 40 per cent. aqueous solution of formaldehyde, and also in the gaseous form for disinfecting rooms. Formalin is used extensively as a preservative and fixative of tissues.

It is important to note that most disinfectants dissolved in absolute alcohol lose much of their bactericidal activity ; this is probably due to the resulting cessation of electrolytic dissociation.

Phenols and Cresols. The coal-tar products are powerful disinfectants. With the exception of phenol they are almost all insoluble in water and form colloidal suspensions. Their activity is considered to be due largely to the penetration of the bacterial cell by the agents in the form of colloidal suspensions, after adsorption on to the surface of the cell.

The preparations in common use are phenol, lysol and tricresol, and of these lysol is the most potent. In laboratories a dish or jar containing a 2 per cent. solution of lysol should always be available to receive contaminated plugs, tubes, pipettes, etc. These substances are frequently added to soaps in order to increase bactericidal action of the latter.

Salts of Heavy Metals. A considerable number of metallic salts possess a marked disinfectant activity, particularly those of mercury and silver. The study of these agents is extremely complicated, but, nevertheless, the following points appear to be well established :—

(1) There are concentrations of salts having respectively stimulating, inhibitory and definitely destructive actions on bacteria.

(2) The toxicity may be due to the undissolved molecule, the anion, the cation or a combination of the three.

(3) Metals with a low atomic weight are generally less toxic than those with a high atomic weight.

(4) The action of the salts is less marked in a protein solution

than in distilled water. This is probably due to the fact that the cation acts on the protein to form an insoluble albuminate.

(5) Organisms vary, but the Gram-positive bacteria tend to be more susceptible to the action of metallic salts than the Gram-negative organisms.

(6) Salts, toxic when acting alone, may become inert in a balanced solution, such as Ringer's solution.

Salts in common use are corrosive sublimate ($HgCl_2$), and complex silver salts, such as protargol and argyrol.

Dyes. Many organic dyes are strong bactericidal agents, *e.g.*, gentian-violet, brilliant green, malachite green and flavine. These have been used for therapeutic purposes, but the results have been disappointing. Gentian-violet has a marked effect on the Gram-positive organisms, but has little action on Gram-negative bacteria. The dyes are frequently used as inhibitory substances in the preparation of selective media ; *e.g.*, brilliant-green for the isolation of Salmonella organisms.

Essential Oils. Essential oils, such as cloves, cinnamon and chaulmoogra oil, have been employed for centuries as preservatives. They have, however, little bactericidal activity.

Process of Disinfection

The mode of action of the various disinfectants is very complex. The principles concerned in the reaction have been subjected to intensive investigations, in which the action of various agents on pure cultures and spores has been tested under controlled laboratory conditions. The process is considered to correspond to a unimolecular reaction and consequently follows the chemical Law of Mass Action ; the reacting substances are the bacteria and the disinfectant. While the process is essentially chemical, physical factors may also influence both components of the reaction.

Disinfection is a gradual process, and with any bacteria a minority will always survive considerably longer than the majority. The greatest number of bacteria is killed at the beginning of the reaction and the death rate decreases as the process proceeds. The velocity of the reaction varies with both the temperature of the reaction and the concentration of the disinfectant. In the case of the temperature, a rise of 10° C. increases the reaction velocity of phenol 8 times and of mercuric chloride 2–4 times. The relationship between the concentration of disinfectant and the rate of disinfection is a logarithmic and not a simple one, and varies with each disinfectant. Thus, doubling the concentration of phenol may increase the reaction velocity 64 times, while doubling the strength of the $HgCl_2$ increases it only twice.

Standardization of Disinfectants. It has already been noted that the reaction between bacteria and disinfectants in the test-tube is subjected to the influence of a number of variable factors. When, however, this interaction takes place in the presence of the body-tissue additional complicating factors are involved. It is thus obvious that the standardization of disinfectants *in vitro* is not likely to give any accurate indication of their activity in the presence of animal tissues. For this reason the tests at present employed for testing disinfectants are by no means ideal.

In testing the *in vitro* efficiency of a bactericidal agent it is necessary to standardize as many factors as possible. The organism under examination must be controlled with regard to age, number and resistance, a fixed time or times of exposure to the agent allowed, and a standard temperature employed for the test. Methods used at the present moment are the Rideal-Walker and the Chick-Martin tests, in which phenol coefficients are obtained.

In the **Rideal-Walker Test** a standard culture of *Bact. typhosum* is tested over given periods (2½, 5, 7½ and 10 minutes) against a 5 per cent. carbolic acid solution and various dilutions of the disinfectant. The **Chick-Martin Test** bears more resemblance to the disinfectant process in nature, in that a fixed amount of organic matter, in the form of dried fæces, is added to the test; yeast is now recommended as a substitute for the dried fæces. A fixed time, 30 minutes, is allowed for this reaction. In both tests a ratio is made of the highest dilution of both agents at which the bacteria are killed under the same conditions.

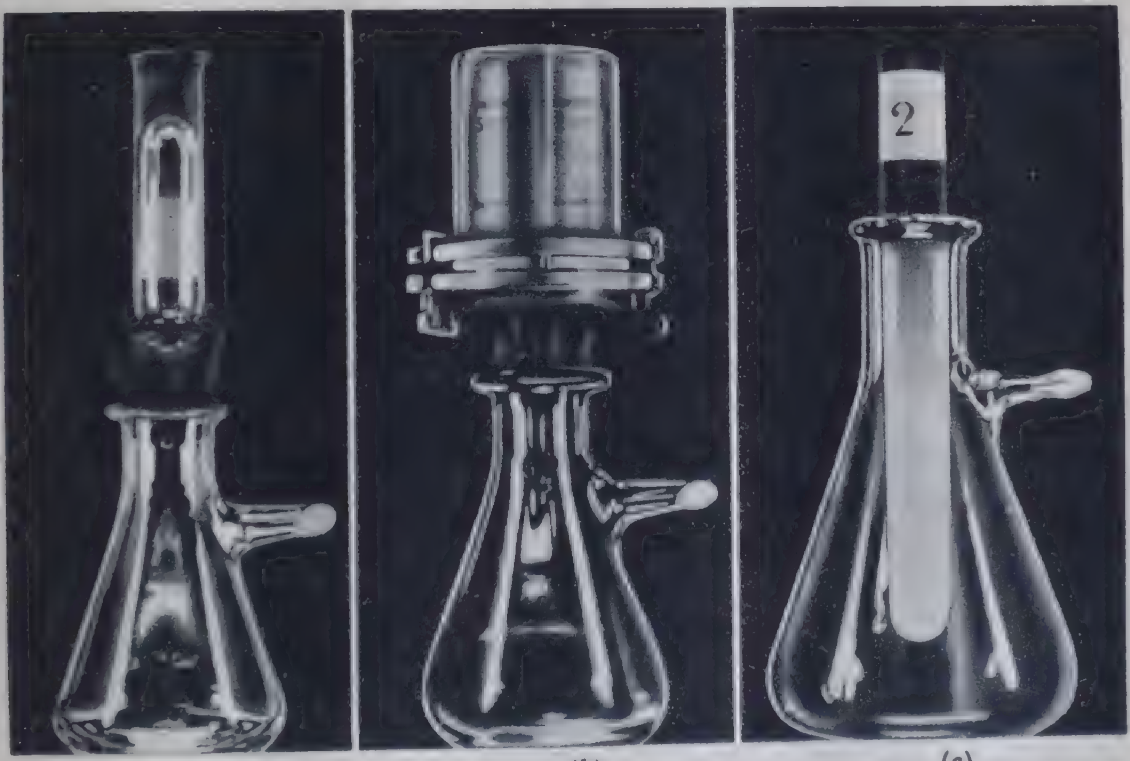
In testing the disinfectant effect on spores, these are dried on threads or garnets, which, after contact with the disinfectant, are well washed and plated out.

The Practical Application of Disinfection. A considerable number of bactericidal agents are available, and in their selection the following points should be remembered. Spores are more resistant than vegetative bacteria; for spores and bacteria with a high lipoidal content, such as the tubercle bacillus, heat is usually more efficient than chemical agents; the higher the temperature of the reaction the greater the efficiency; disinfectants tend to lose their bactericidal activity when dissolved in alcohol or vegetable oils; the presence of organic matter, especially protein, lowers the activity of most disinfectants.

The following properties are desirable in any disinfectant: low tissue toxicity, stability, lack of odour, low cost, solubility, power of penetration, and action in the presence of organic matter. It must, however, be noted that in some instances where disinfectants are used, *e.g.*, in mouth washes, gargles, toothpastes, etc., they are not likely to have much effect *per se*, the main action is that of cleansing the infected region.

Removal of Bacteria by Filtration

Filtration is frequently carried out to remove bacteria from solutions, and for this purpose various types of filter are available. Filtration is widely employed in the sterilization of substances that are liable to be damaged by heat; it is thus used in the preparation of toxins, serum and ascitic fluid media, certain broths, and in the study of filterable viruses.



(*a*) (*b*) (*c*)

FIG. 11.—Bacterial filters—(*a*) Berkefeld, (*b*) Seitz, and (*c*) Chamberland (L2)—fitted into flasks provided with a side tube, which is connected with the exhaust pump.

Three main types of bacterial filter (Fig. 11) are in use:—

(1) Diatomaceous earth or kieselguhr filters.

(2) Chamberland filters.

(3) Seitz filters.

(1) The two best-known types of diatomaceous earth filters are the Berkefeld and the Mandler. The Berkefeld is the original German filter and is made from sifted kieselguhr mixed with asbestos and organic matter. This filter is arranged in the form of a small candle and is prepared in three grades :—

(i.) " V," the coarsest grade, will not hold back the smaller bacteria, such as *Chr. prodigiosum.*
(ii.) " N," the intermediate grade, is the common type in this country ; it does not allow bacteria to pass through.
(iii.) " W " is the finest grade.

As the individual filters of the various grades vary considerably in their pore size, any comparison of the results obtained by different workers using filters of the same type is therefore not practicable. These filters are good, but, as they are extremely fragile, they require careful handling. The Mandler is an American modification of the Berkefeld.

(2) Chamberland filters or bougies are unglazed porcelain candles used extensively in France. They are made in nine grades of porosity : L1, L1 bis, L2, L3, L5, L7, L9, L11, L13—the L1 candle is the coarsest grade and the coarseness decreases through the intermediate grades with the L13 candle the finest.

(3) Seitz filters are asbestos discs of varying diameters (3 cm., 6 cm. and 14 cm.) and are made in two grades of porosity : " K " for clarifying, and " EK " for removing bacteria. These filters are used on a large scale at the present time ; they are both efficient and easy to set up ; as a new disc is employed for each filtration, the problem of cleaning is obviated.

In filtration the material is drawn through the filter by pressure from a vacuum pump ; a negative pressure of 100–200 mm. of mercury is sufficient for ordinary purposes. After use the components of the filter should be sterilized and cleaned ; candles should be rubbed with a stiff brush and then trypsinized or boiled in 2 per cent. sodium carbonate solution and finally washed well in water.

Filtration is an exceedingly complicated process, involving a consideration of many factors. It is by no means a simple mechanical procedure governed only by the relative sizes of the pores of the filter and the particles to be filtered. The most important factors implicated are : the composition and electrical charge of the filter, the *p*H and electric charge of the material undergoing filtration ; the amount of protein or other extraneous material in the suspension ; the duration of filtration ; the temperature at which the process is carried out ; the pressure employed. Adsorption plays an important *rôle* in filtration, particles may be adsorbed either on to the filter or on to albuminous material present in the suspension, and so fail to pass through a filter. In recording results of filtration it is therefore necessary to give complete details of the technique employed.

Ultrafiltration is considered later in the section on viruses (p. 388).

CHAPTER VII

CHEMOTHERAPY

THE production of arsphenamine (606) by Ehrlich in 1910 and its success in the treatment of syphilis raised hopes that other bacterial diseases would respond to chemotherapeutic agents. In the following years many attempts were made to treat bacterial diseases by chemotherapy but, except in the case of spirochætal diseases, the results were disappointing and this form of therapy had been practically abandoned until the recent introduction of the sulphonamide compounds. Chemotherapy has, however, been widely employed in many protozoal diseases, *e.g.*, quinine in malaria, arsenical compounds in trypanosomiasis, antimony preparations in schistosomiasis and leishmaniasis.

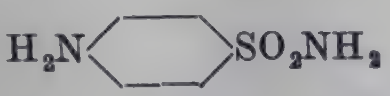
In 1935 the position was completely revolutionized by Domagk's demonstration that streptococcal infections could be successfully treated by a sulphonamide preparation named Prontosil. A considerable amount of research has since been carried out on the sulphonamide group of drugs ; the early results have been confirmed and elaborated and there is now little doubt that this group has proved one of the most valuable therapeutic agents introduced into medicine.

A further striking advance was the production of penicillin in a concentrated and stable form by Florey, Chain and their co-workers in 1940. Penicillin has since been used extensively in the therapy of war-wounds and many diseases with great success. Numerous attempts have been made, with varying degrees of success, to prepare similar substances from other moulds and bacteria. Chemotherapeutic agents of this type have been termed " antibiotics."

The Sulphonamides

Sulphonamide preparations were used as dyes some 30 years ago, but they were not considered as therapeutic agents until Domagk reported the excellent results given in streptococcal infections by prontosil, a red diazo-dye. These observations were soon confirmed by independent workers in all parts of the world. Much research was consequently stimulated with a view to extending the scope of this new group of chemotherapeutic agents and a considerable amount of literature has accumulated on the subject.

It was soon proved that the active substance in the complex dye, prontosil, was a relatively simple substance, **sulphanilamide** or *p*-aminobenzenesulphonamide, which has the following structural formula :—



It was also found that sulphanilamide has a limited range of activity for bacteria ; sensitive organisms include *Strept. hæmolyticus*, gonococcus, meningococcus, and *Bact. coli.* In order to extend the range of these chemotherapeutic agents, numerous derivatives, probably thousands, have been synthesized, but only a few have been satisfactory. The majority of the new compounds proved either inactive or too toxic to be of any practical value in the treatment of disease. Where, however, the resulting compounds have been satisfactory, they have been given individual trade names. This has given rise to a chaotic state, in which similar derivatives are sold under different names ; for example, prontosil album, streptocide, proseptine, colsulanyde, sulphoamide P, to name only a few, all have sulphanilamide as the active principle, and consequently all have a similar range of antibacterial activity ; any differences in these products are usually in degree of solubility and toxicity. An attempt is now being made to reduce the confusion caused by this multiplicity of trade names by the adoption of a standard nomenclature for these drugs.

Numerous modifications of the sulphanilamide molecule have been produced by replacing one or more of the hydrogen atoms with other groups ; the only derivatives, having increased activity and so greater therapeutic value, have been obtained by the substitution of one of the hydrogen atoms of the SO_2NH_2 or amide group. Unlike prontosil, these substances do not break down to sulphanilamide but act as the whole molecule.

The first of these compounds was **sulphapyridine,** which, in addition to covering the same bacterial range as sulphanilamide, usually with greater effect, is also active against pneumococci of all types. Absorption may be irregular and unpleasant symptoms, such as nausea and vomiting, are not uncommon. Additional compounds have since been produced which, although similar in bacterial range, differ by varying degrees in their solubility, rate of absorption, diffusibility, rate of excretion, and toxicity. These include **sulphathiazole, sulphadiazine, sulphamezathine** (sulphadimethylpyrimidine or sulphamethazine), **sulphamerazine,** and **sulphapyrazine.**

Sulphaguanidine, succinylsulphathiazole (sulphasuxidine) and **phthalylsulphathiazole** (sulphathalidine) are poorly absorbed from the intestines and have proved valuable in the treatment of bacillary dysentery.

A German product, **marfanil,** is active in the presence of pus : claims that it is particularly active against the anaerobes have not been substantiated. **Promin,** a sulphone, has been used for the treatment of tuberculosis ; the initial results have not been very encouraging.

Pharmacology. Sulphonamides may be administered by three routes : (1) oral, (2) parenteral, intravenous or intramuscular, and (3) local. The oral route is usually employed, but parenteral injection is necessary in severe, fulminating infections when an immediate action is required, or for patients not tolerating oral administration. For this purpose sodium salts of the different derivatives are used as these are more readily soluble in water. These preparations are alkaline (*p*H more than 9.0) and they may cause pain and possibly necrosis at the site of injection. For these reasons they should never be given intrathecally.

Sulphonamides may be used locally in the form of powders, solutions or ointments. In the prophylaxis of war wounds a powder containing penicillin and a sulphonamide proved a great success.

With few exceptions, *e.g.*, sulphaguanidine, succinylsulphathiazole and phthalylsulphathiazole, the sulphonamides are rapidly absorbed from the alimentary tract, mainly from the small intestine. The absorption of sulphapyridine tends to be slower and less regular than that of comparable compounds ; sulphadiazine is also more slowly absorbed than sulphathiazole or sulphamezathine. The sulphonamides readily diffuse and soon after administration they are present in the tissues and body fluids ; the rate varies with the different derivatives and appears to depend on the extent to which they become bound to the plasma proteins and so are unable to dialyse. This combination is not very firm and soon breaks down.

Excretion occurs soon after the administration of the drugs and is mainly by the urine, the drug is found either in the free form or as an inactive acetyl compound which is much less soluble than the parent sulphonamide. The conjugated forms thus tend to cause urinary symptoms by forming crystals which block the renal tubules, pelvis or ureters.

The dosage of the drugs is governed by these facts. It is essential to maintain an effective concentration of the sulphonamides in the tissues for several days and, because of their rapid excretion, regular dosage at short intervals is essential. A common routine course in severe adult cases is to give 2 or 3 gm. orally at once and then 1 gm. at 4-hourly intervals and to maintain this for several days, when, if the response is good, the dose is decreased until recovery is complete. The drugs are usually prepared in tablets of 0·5 gm.; these should be powdered and administered in milk or water. It is important that there should be no break in the 4-hourly intervals, patients should be awakened if necessary, as irregularity of administration may cause the blood concentration to fall below the effective level. An attempt is usually made to maintain a concentration of some 8–12 mgrm. of free sulphonamide per 100 ml. of blood during the initial stages of treatment, but whether such a relatively high concentration is always essential has not yet been ascertained.

It is important that, whenever possible, the administration of the sulphonamides should be subjected to bacteriological control. The various compounds vary in their action on different bacteria and, unless the causative organism is identified, patients may be submitted to prolonged dosage with an inactive preparation. If the results with a particular sulphonamide have not proved satisfactory, further treatment should be carried out with another type of preparation; the substitution of one sulphanilamide preparation for another of a different trade name is without value as these have a similar range of antibacterial activity.

Virus infections, with the probable exception of lymphogranuloma venereum, have not proved susceptible to the action of the sulphonamides.

Tentative treatment with small amounts of a sulphonamide is to be condemned at all times, as the concentration reached under such conditions is too low to be of value and there is the important possibility of rendering susceptible bacteria drug-resistant. The use of small doses of a sulphonamide as a prophylactic measure against upper respiratory infections, cerebrospinal fever and bacillary dysentery is not recommended except in very special circumstances. As a routine measure it may lead to prolonged administration of small amounts of the drug. This may not only produce resistant strains of normally sensitive bacteria but also lead to the development of sulphonamide-sensitivity by the patient. It is also possible that, in the case of upper respiratory infections, the causative organism may be sulphonamide resistant, *e.g.*, a virus.

In order to avoid the onset of renal complications, large amounts of alkaline fluids should be taken during intensive dosage with the sulphonamides.

Toxicity. The sulphonamides are not tolerated equally well by all individuals, and in susceptible persons a wide range of toxic manifestations may be produced, particularly during intensive dosage. There is also some difference in the degree of toxicity of the various sulphonamides. The toxic symptoms resulting from the administration of the sulphonamides may be either mild or severe.

Mild symptoms are usually nausea, vomiting, cyanosis, rash, headache and hæmaturia. These generally clear up quickly if copious fluids are administered and the drug is stopped or the dose decreased. If it is necessary that treatment should be continued, penicillin or some other form of effective sulphonamide should be given.

Severe symptoms are agranulocytosis, hæmolytic jaundice and anuria. These are fortunately rare, but when present they require urgent treatment. The drug should be stopped immediately and copious alkaline fluids administered ; in the case of agranulocytosis or hæmolytic jaundice, blood transfusion may be necessary. Anuria is caused by the formation of acetyl crystals in the renal tubules, the pelvis of the kidney or the ureters and is particularly liable to occur in a very acid and concentrated urine. If this condition does not settle down after the intake of copious fluids, ureteric catheterization may be necessary. In order to avoid serious complications large doses of sulphonamides should not be given to patients with impaired renal function, while regular blood counts should be carried out when intensive courses are given.

Mode of Action. Much research has been carried out to determine the mechanism of action of the sulphonamides. It was soon discovered that these compounds, *per se*, had very little bactericidal activity and also that they caused no increase in the antibacterial properties of the leucocytes. Investigations, carried out mainly on the sulphanilamide preparations, indicate that these drugs act by interfering with the normal metabolism of the bacteria, preventing growth and so rendering them a ready prey for the normal defence mechanism of the body. The action of sulphonamides is thus essentially bacteriostatic.

It has already been pointed out that, during bacterial multiplication, the bacterial protoplasm must be synthesized by enzyme action from substances present in the medium and that bacteria differ considerably in their power to synthesize these complex proteins (p. 26). As an illustration it has been found that some bacteria, *e.g.*, *Bact. coli*, are able to multiply with ammonia as the source of nitrogen while others, *e.g.*, *Bact. typhosum*, require the addition of certain amino-acids before growth can take place. Proceeding further, organisms such as the staphylococcus, require more complex substances, *e.g.*, nicotinic acid, in order to build up their protoplasm.

Fildes has given the term "essential metabolite" to the substance or chemical group which takes an essential part in the chain of synthesis necessary for bacterial growth. A "growth factor," which must be added to the medium in order to obtain bacterial multiplication, is an essential metabolite which the cell cannot synthesize. Fildes considers that the sulphonamides act by competing for an enzyme associated with an essential metabolite, thereby interfering with the bacterial metabolism and preventing growth. In the case of the sulphonamides the indications are that the essential metabolite is *p*-aminobenzoic acid; sulphonamides are able to combine with the same enzymes as *p*-aminobenzoic acid, but are sufficiently different in structure to be devoid of essential metabolic activity. Bacterial multiplication is consequently inhibited and, as a result, the normal defence mechanism is rendered more effective and is able to overcome the invading organisms.

A practical outcome of this work is the addition of *p*-aminobenzoic acid (5 mgrm. per 100 ml.) to media used for culturing material collected from patients receiving sulphonamide therapy. In this way any inhibitory action of the sulphonamide is eliminated.

This concept of bacterial metabolism opens up an important field for the biochemist. A knowledge of the synthetic activity of the bacteria makes possible the deliberate preparation of substances related to essential metabolites; these compete for the associated enzyme and so interfere with metabolism and exercise a bacteriostatic action. The following is an illustration of such a planned compaign. Pantothenic acid was known to be an essential factor for *Streptococcus hæmolyticus*, and it was later found that on addition of pantoyltaurine to the medium growth of this organism was inhibited. A trial of pantoyltaurine in experimental streptococcal infections was then made with successful results.

Antibiotics

It has long been known that bacteria and moulds produce substances injurious to other organisms and naturally many attempts have been made to separate these products and utilize them for therapeutic purposes. The results were, however, unsatisfactory until the recent work of Florey and his co-workers on penicillin, which has proved a most valuable chemotherapeutic agent.

At one time or another antagonistic substances have been obtained from many organisms including *Ps. pyocyanea*, *B. subtilis*, *B. brevis*, *Actinomyces*, *Streptothrix* and moulds. The name " antibiotic " has been given to these antibacterial products of bacterial or fungal growth, of which by far the most important is penicillin.

Penicillin

The history of penicillin starts with the observation by Fleming (1929) that, on an agar plate of staphylococcus contaminated with a mould, the colonies in the neighbourhood of the contaminant had faded. He investigated this phenomenon in detail and found that the mould, *Penicillium notatum*, produced a soluble substance, *penicillin*, which had a marked inhibitory action on many organisms besides the staphylococcus. This penicillin proved weak and labile and attempts to concentrate it for therapeutic use were unsuccessful.

Fleming however used it in the preparation of a selective medium for the isolation of *H. influenzæ*, which was not inhibited by weak solutions of penicillin.

Further progress was not made until Florey, Chain and their co-workers (1940) prepared penicillin in a concentrated and relatively stable form. They were consequently able to demonstrate the therapeutic properties of this antibiotic in experimental infections with streptococci and staphylococci ; tests on human infections, particularly with the staphylococcus, were also a great success.

Large-scale production was then commenced, first in America and later in this country, and penicillin has since been extensively used with striking success in the treatment of war-wounds and many infections.

Properties. Penicillin is obtained from cultures of the mould *Penicillium notatum.* Synthesis of penicillin has not yet been reported, although its chemical structure has been determined; it is a special form of dipeptide, and four different penicillins have been identified.

In order to get the maximum production of penicillin special media and incubation at 24° C. are necessary. Deep-cultures have now replaced "surface" techniques. Stock cultures of *Penicillium notatum* should not be maintained on the usual media ; potato starch-agar and molasses-agar are recommended ; sub-culture should be carried out as infrequently as possible.

Penicillin is an acid ; it is unstable and is readily destroyed by mineral acids, alkalies, heavy metals, alcohols, oxidizing agents and heat over 40° C. It is also inactivated by many forms of synthetic rubber and consequently all rubber used in a continuous drip apparatus must be tested before use and only satisfactory tubing used. Some bacteria, *e.g.*, coliforms and *B. subtilis*, produce an enzyme, *penicillinase,* which rapidly destroys penicillin.

Penicillin for therapeutic use is supplied in the form of the calcium or sodium salt, both of which are highly soluble, relatively stable between pH 5 and 7, and are issued either in tablet form or as a powder. Such preparations, however, contain only 20–60 per cent. of pure penicillin, the remainder being impurities which may give rise to unpleasant reactions on parenteral inoculation. The sodium salt is hygroscopic, but the calcium is practically non-hygroscopic and therefore is frequently used in the preparation of powder for local application. The potency of penicillin is assayed by biological methods and is expressed in terms of the arbitrary Oxford unit. This unit has been accepted as an International Standard and is defined as the activity contained in a certain weight of a sample of penicillin selected as a standard of reference. Potency is thus usually expressed in terms of "X" units per mgrm. Another term "megaunit" is frequently used and represents one million units.

The common pathogens vary in their sensitivity to penicillin in the usual therapeutic concentrations. Their behaviour is indicated in the following list :—

ORGANISMS SENSITIVE TO PENICILLIN. *Staph. pyogenes, Strept.*

hæmolyticus, *Strept. viridans*, pneumococcus, gonococcus, meningococcus, *B. anthracis*, *C. diphtheriæ*, *Cl. welchii*, *Cl. œdematiens*, *Cl. septicum*, *Cl. tetani*, *Actinomyces*, *Trep. pallidum*, *Lept. icterohæmorrhagiæ*.

Organisms Relatively Insensitive. *Bact. typhosum*, some of the Salmonella group, *Strept. fæcalis*, *H. influenzæ*.

Organisms Insensitive. *Bact. coli*, *Ps. pyocyanea*, *H. pertussis*, *Proteus*, *Br. abortus*, *Br. melitensis*, *Past pestis*, Friedlander's bacillus, tubercle bacillus, viruses.

Thus Gram-positive organisms and Gram-negative cocci tend to be highly sensitive, whereas Gram-negative bacilli and viruses tend to be resistant. It is therefore important that penicillin therapy should always be subjected to bacteriological control.

Penicillin is almost completely non-toxic and it is also effective in the presence of pus, blood and serum. It has thus many advantages over the sulphonamides. The mode of action of penicillin is not known. It is effective in very high dilutions; with the purer forms a dilution of 1 in 50 million or more will inhibit some organisms. The action is both bacteriostatic and bactericidal. Penicillin frequently interferes with the power of bacteria to divide and large grotesque forms of the organisms develop. If, however, it is added during the logarithmic or dividing stage of bacterial growth, some organisms are rapidly lysed.

Methods of Administration. Oral administration is not yet possible as the acid of the gastric juice destroys penicillin. Many attempts have been made to overcome this difficulty but, as yet, without any great success.

Penicillin may be given either (1) parenterally or (2) locally.

(1) **Parenteral Administration.** The methods used for systemic treatment are either intermittent injections or continuous infusions. The injection can be given by the intramuscular or intravenous route; the former is the method of choice as penicillin tends to cause thrombosis. Penicillin on introduction into the tissues diffuses widely, but it is rapidly excreted by the kidneys and heavy dosage is therefore necessary. As in the case of sulphonamide therapy, it is essential that an effe[illegible]e concentration of penicillin should be maintained in the tissues during the course of treatment. It does not, however, reach serous and synovial cavities, and therefore in pleural, meningeal and joint infections, injections should also be given into the affected region to reinforce the systemic inoculations.

The usual dosage is 20–25,000 units at intervals of three hours ; a useful strength for the solution is 10,000 units per ml. It is important that syringes should be heat-sterilized, as many chemical disinfectants, *e.g.*, methylated spirits, destroy penicillin. When the continuous drip method is used, some 150,000 units are added to 1–2 pints of normal saline for each period of 24 hours ; satisfactory rubber tubing must be used. Aseptic precautions must be observed and administration is continued until the clinical response is satisfactory.

Intermittent injection for several days at 3-hourly intervals is a tedious business and attempts are being made to use slow-release vehicles, such as pea-nut oil and beeswax. By such means it is hoped that the interval between injections can be prolonged and an effective concentration of penicillin in the blood still maintained.

(2) **Local Application.** Penicillin may be used locally in many ways :

(*a*) As a **powder** ; the calcium salt is mixed with some sulphonamide to give a final strength of 5,000 units of penicillin per gramme, this can be further diluted if necessary ; such a powder is valuable in treating wounds or skin grafts ; an insufflator is necessary.

(*b*) In **solution,** usually 500 units per ml. of sterile water or normal saline ; the solution must be stored in the refrigerator.

(*c*) As an **ointment** with lanette wax, 500 units penicillin per gramme ; if kept in a refrigerator potency is retained for 3–4 weeks.

(*d*) In **lozenges** or **pastilles**, sometimes made with a gelatin base, each pastille containing 500 units of penicillin. These have proved valuable in such infections as acute ulcerative gingivitis (Vincent's angina) and acute tonsillitis ; the pastilles, if not chewed or sucked, should last 1 to 2 hours ; when dissolved a fresh pastille should be given.

(*e*) In **lamellæ** containing 500 units in each ; these have given good results in infections of the conjunctiva and cornea.

Penicillin is in the early phases of development and many problems await solution, *e.g.*, the preparations at present available for therapeutic purposes contain impurities ; penicillin cannot be given by oral administration ; synthesis is not yet possible. Because many bacteria are resistant to penicillin, research is now being carried out on other antibiotics, such as *gramicidin*, *streptomycin* and *streptothricin*. It is thus possible that some new substance having a wider and more powerful action will be obtained either from a mould or bacterium or as a result of synthesis of penicillin and subsequent modification of the molecule, as has been done with sulphanilamide.

Laboratory Control of Penicillin Therapy. Laboratory methods are necessary (1) to determine the sensitivity of the infecting organisms, (2) to carry out penicillin assays, and (3) to ensure that an adequate level of penicillin is being maintained in the blood.

(1) The *bacterial sensitivity* may be tested by adding a small amount of penicillin to an agar- or blood-agar plate seeded with the organism to be tested. Penicillin can be added by (*a*) the *punch hole* method—holes are bored into the medium by means of a No. 5 cork-borer and penicillin, 1 and 10 units per ml., added to different holes ; (*b*) *cylinder* method—special assay cylinders of porcelain are dropped on the medium and filled with weak penicillin solutions (Fig. 12) ; (*c*) *filter-paper* method—strips or discs of sterile filter paper are soaked in penicillin, 20 units per ml., and then laid on the dried surface of the plate ; (*d*) ditch-plate method—a strip is cut out of an agar plate and is replaced by agar containing penicillin (about 10 units per ml.) ; stroke cultures are then made up to the ditch. In all cases the penicillin diffuses into the surrounding medium and a zone of inhibition indicates sensitivity.

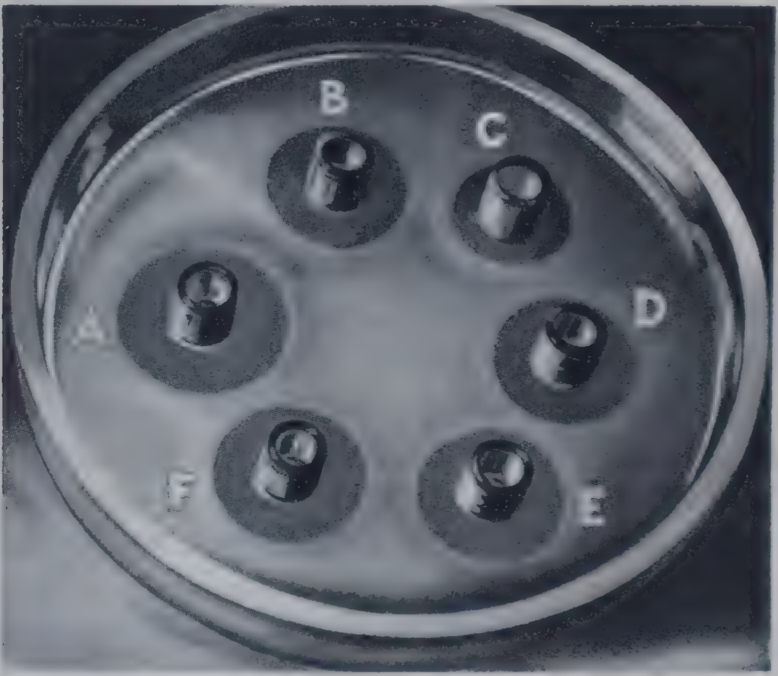


Fig. 12.—*Penicillin Assay.* Cylinder method, with 2, 1 and 0·5 units of penicillin per ml. in cylinders A, B and C respectively and the unknown in cylinders D, E and F.

Another method is to prepare serial dilutions of penicillin in broth and to add a standard loopful of the organism to be tested; incubate for 24 hours at 37° C. and read. A control series with the standard Oxford staphylococcus should always be prepared; this organism is generally inhibited by 0·01–0·02 units of penicillin per ml.

(2) *Penicillin Assay.* This may be carried out by the methods described above, except that the unknown quantity is now the penicillin and not the organism, which in this country is the Oxford Staphylococcus H strain. The "cylinder" method is frequently employed, six cups to a plate; dilutions of standard penicillin, 2, 1 and 0·5 units per ml., are added to three cups and appropriate dilutions of the material to be tested are added to the remaining three cups (see Fig. 12). After overnight incubation zones of inhibition are noted and compared; the penicillin content of the unknown material can then be determined. Another satisfactory method is simple titration in broth. Falling dilutions of standard penicillin and the test material are made in broth and then each is seeded with a fixed amount of the standard staphylococcus. By comparing the inhibition after incubation, the potency of the unknown can be calculated.

(3) *Penicillin Content of Blood.* It is frequently important to know the penicillin level of the blood of patients receiving treatment. Methods for this purpose usually involve the use of slide-cells or some similar device. In one method small numbers of the standard staphylococcus are added to varying dilutions of the patient's serum on a slide, sterile cover glasses are added and the edges sealed. After incubation the number of colonies growing are counted and the presence of inhibition noted. Another method is to add blood to the medium and use *Strept. hæmolyticus* as the test organism; this can be set up in capillary tubes as well as in slide-cells; the end-point can be detected after overnight incubation, and is shown by absence of hæmolysis. The dilution method in broth can also be used, but this requires more serum; the Oxford Staphylococcus or *Strept. hæmolyticus* can be used as the test organism.

CHAPTER VIII

INFECTION

While bacteria are responsible for many diseases of man and animals, it is, however, important to recognize that relatively few bacteria are capable of producing disease ; the majority are unable to multiply in the tissues and are readily disposed of by the normal defence mechanism of the body. Bacteria, as previously indicated, have been grouped into two arbitrary classes —the *parasites* which grow upon living tissues and the *saprophytes* which multiply on dead organic matter. The differentiation of these two classes is not absolute, since there is considerable overlapping by members of both groups. Another tentative but convenient classification is the sub-division of bacteria into those able to produce disease—the *pathogenic* forms, and those without this capacity—the *non-pathogenic* forms. Again the terms are only relative, as an absolute division into two such groups is impossible. This may be appreciated from the fact that one bacterium is not infrequently pathogenic for one animal species and not for another. Also many so-called non-pathogenic bacteria may produce pathological changes if sufficient numbers are introduced into the tissues.

The multiplication of a bacterium in the tissues with consequent damage, no matter how slight, to the host, is termed *infection*. The mere presence of a pathogenic organism in the tissues does not necessarily constitute infection ; penetration through either the skin or mucous membrane and subsequent multiplication must take place. The presence of infection is sometimes not clearly indicated, for example the entrance into the tissues of a small number of bacteria may lead to the production of immunity without the appearance of any definite clinical manifestations ; this is termed *subclinical or latent infection*. In other cases the multiplication of bacteria in the tissues may give rise to lesions not characteristic of infection with the particular organism ; this is known as an *atypical infection*.

The term *infectivity* denotes the power of a particular strain to spread from host to host, and induce some form of infection.

The surfaces of the body, *e.g.*, the skin and mucous membranes, normally harbour a number of bacteria, which multiply without damage to the host: these are termed *commensals*. These organisms are strictly saprophytic, as their food supply is not obtained at the expense of the living cells, but is derived from the local secretions, *débris*, excretions and other foreign matter. Many varied bacterial species come in contact with the body surfaces, but only a few find the conditions suitable for multiplication. The flora of the different regions, while not absolutely constant, tends to maintain a characteristic distribution, which is largely dependent on the environmental conditions. The upper respiratory tract usually harbours various strains of streptococci, Gram-negative cocci, staphylococci, diphtheroids, and pneumococci. The alimentary tract has a particularly varied flora, which can be modified by dietetic changes, *e.g.*, proteolytic organisms are predominant when a protein diet is taken. The vaginal secretion is acid and aciduric bacteria, such as the lactobacilli, are generally present. The regions in close contact with the air are seldom, if ever, free from bacteria under normal conditions. In contrast to this, the tissues having more remote contact with the outside world, such as the uterus, bronchioles, lungs, sinuses and the middle-ear, do not normally harbour organisms.

Many commensals are potential pathogens, and on any decrease of the body resistance they may give rise to disease. Thus the pneumococcus (Group IV) is usually present as a member of the normal flora of the naso-pharynx; when, however, the resistance of the body is lowered, as after or during a debilitating illness such as measles or influenza, a secondary pneumococcal bronchopneumonia not infrequently develops.

The active pathogens tend to be more strictly parasitic and develop mainly, in some cases solely, in the body-tissues. Their presence in the tissues is therefore generally associated with a disease process. In many instances the resistance of the community to these organisms is low, and when the chances of infection are high, extensive outbreaks or epidemics occur, as in the case of diphtheria, measles, influenza, etc.

Transmission of Infection. The manner in which an infection is transmitted constitutes a factor of great importance in determining not only the probable extent of an outbreak in a community but also the organization of suitable prophylactic measures. The ultimate sources of infection are almost invariably the excreta and discharges of other individuals or animals. The spread of infection may occur in many ways, which can be conveniently arranged in four classes :—

(1) by direct or indirect contact,
(2) by air-borne or aerial transmission,
(3) by ingestion,
(4) by insects.

This is merely an arbitrary arrangement as there is considerable overlapping of these classes ; all are forms of direct or indirect contact, the main differences being the portal and method of entry, viz., by inoculation, inhalation or ingestion.

(1) Transmission of infection by *direct contact* is common, but epidemics are not produced in this manner as personal contact with an infected individual is necessary. Important examples of this type of infection are the venereal diseases. Rabies, some cases of anthrax and psittacosis are instances in which infection results from direct contact with infected animals and birds.

Spread by *indirect contact* is also common. Actual personal contact is not involved, the infection is conveyed by some inanimate object. Examples are the various infections resulting from the handling of contaminated swabs, instruments and clothes, or the use of contaminated drinking vessels or shaving brushes. In some cases the organisms are implanted directly into the tissues as a result of trauma as in post-mortem infections in which the organisms, often *Strept. hæmolyticus*, are introduced through accidental incisions, and in gas-gangrene and tetanus in which there is contamination of the wound with soil and manure. In Weil's disease, the causative organisms are excreted in the urine of infected rats and reach stagnant water from which they pass into the human tissues *viâ* superficial scratches and abrasions.

(2) Diseases of the upper respiratory tract, such as diphtheria, whooping cough, scarlet fever and measles, are usually passed from man to man by *aerial transmission*. Numerous organisms are sprayed into the surrounding atmosphere by such acts as coughing and sneezing. Some of these are contained in relatively large droplets which tend to fall rapidly and so are a source of infection only for other persons in the immediate vicinity. Smaller particles, which are termed droplet-nuclei, can, however, remain suspended in the air for relatively long periods of time, sometimes for several hours, and are readily transported by air-currents. Particles falling to the ground become dried and resistant organisms remain viable in the dust, which thus become

a source of infection; tubercle bacilli, diphtheria bacilli and *Strept. hæmolyticus* have been isolated from the dust of wards and sick rooms. In crowded rooms with poor ventilation the bacterial content of the air is high and the chances of spreading infection are consequently great. Diseases of this group are frequently encountered in an epidemic form.

(3) Spread of infection by *ingestion* is found most frequently in the intestinal diseases, such as enteric fever, dysentery, food poisoning, cholera, etc. The vehicles are food, milk and water which have become contaminated with the excreta of cases or carriers. Extensive outbreaks may result if large numbers have consumed the contaminated food or drink. Bovine tuberculosis, undulant fever and abortus fever are transmitted by the ingestion of raw milk collected from infected animals. Some cases of Salmonella food-poisoning have been caused by the contamination of food with the fæces of infected rodents. Flies and other insects may also act as agents for the transmission of infection by transferring, on their pads, pathogens from excreta to food.

(4) There are many diseases in which infection does not usually take place directly from man to man, but is transmitted by the agency *of insects*. An infected individual is bitten by a blood-sucking insect, which harbours the responsible organisms and subsequently implants them into the tissues of healthy individuals. Important diseases in this category are yellow fever, typhus fever, plague and sandfly fever, which are transmitted respectively by the mosquito, the louse, the flea and the sandfly. Malaria, a protozoal disease, is a further important example. The incidence of these diseases is obviously dependent, to a great extent, on the distribution of the insect host. Thus yellow fever is found mainly in tropical regions, while plague and typhus fever are usually encountered in countries where hygienic conditions are poor.

A knowledge of the methods of disease transmission is of great importance in the practice of epidemiology, hygiene and public health. It is possible either to limit or to exterminate a disease by reducing or removing its vehicles of infection. Rabies has been eradicated from this country as a result of the passing of muzzling and quarantine regulations; attention to drinking water and sewage disposal has eliminated cholera and greatly reduced the incidence of enteric fever in this country; hospitalization and quarantine have likewise reduced the morbidity rate of the acute exanthemata; tuberculosis of bovine origin has been checked by the stricter control and pasteurization of milk;

insecticides, such as D.D.T., have recently played a big part in the control of typhus fever ; measures to control infections of the upper respiratory tract include good ventilation, air-conditioning, avoidance of over-crowding, the use of the handkerchief while sneezing and coughing, the treatment of the air by ultra-violet radiation or by fine mists or aerosols, and the suppression of dust by certain oils.

Infection almost invariably originates from either a human or an animal source and the greatest danger to the community is not the frank case, which is easily diagnosed and controlled, but the unrecognized case and the carrier. Atypical infections are frequently undiagnosed in the early stages, during which the infecting agent may be widely dispersed.

The **carrier,** who is an apparently healthy individual harbouring a pathogenic organism, is an important source of infection. Carriers are a potential and real danger to the community and they constitute a serious problem to the public health officials, as they are not only difficult to detect but also refractory to treatment. Detection is only possible by laboratory methods. The carrier-state is by no means uncommon ; it may succeed a definite attack of the associated disease or may develop in an apparently healthy individual. In this country the carrier is responsible for outbreaks of many diseases, including diphtheria, enteric fever, dysentery, meningococcal meningitis and poliomyelitis.

In view of the great increase in air transport, a problem now confronting the public health officials is the possibility of the entrance into the country of infected but symptomless individuals. *i.e.*, during the incubation period, into uninfected regions.

The *rôle* played by bacteria in the production of disease was conclusively demonstrated by Pasteur, Lister and Koch in the second half of the last century. A new field of research was opened and many workers were attracted. As a result much work was accomplished, but unfortunately a considerable amount was of an unsatisfactory nature. Bacteria were readily isolated from the body-tissues and many extravagant claims made of their relationship to disease. In view of this unsatisfactory state of affairs Koch, in various communications, formulated his now-classical postulates on the requirements necessary to incriminate bacteria as the causative agents of disease. Koch emphasized that an organism, before it could be considered the ætiological agent of a disease process :—

(1) should always be associated with the disease,
(2) must be isolated in pure culture,
(3) should, in pure culture, reproduce the disease in a healthy susceptible animal, and
(4) must be isolated in pure culture from the lesions of the inoculated animal.

Attention to these criteria has undoubtedly had a definite beneficial effect on experimental work, serving to check unsatisfactory claims of causal relationship to disease made for numerous organisms, which were merely contaminants or secondary invaders. The ubiquity of bacteria and the comparative ease with which many non-pathogenic forms can be cultivated are frequently responsible for contamination during the examination of the various diseased tissues. While all the criteria laid down by Koch cannot be fulfilled in every case they undoubtedly form an excellent working base-line. Difficulties are not infrequently encountered : for example there is no animal, apart from man, susceptible to infection with the gonococcus ; some filterable viruses and the leprosy bacillus cannot be readily cultivated *in vitro ;* viruses cannot be detected by ordinary microscopic methods. These facts do not, however, detract materially from the value of the postulates and, before any claims concerning the relationship of a bacterium to a disease are made, a fulfilment of the criteria should be attempted. Further assistance in establishing the causal relationship of an organism to a disease process is now obtained in some cases from serological tests and the use of skin reactions.

Infection is the result of an interaction between the invading bacterium and the body-tissues in which the tissues suffer damage. When the bacterium is particularly active or virulent and the resistance offered to the invasion is of a low order, the result is a generalized fulminating infection, which frequently ends in death. On the other hand, invasion of resistant tissues by an organism of low virulence tends to result in either a localized lesion or merely subclinical infection. Between the two extremes, *i.e.*, a generalized fulminating infection resulting in death and subclinical infection, a wide range of reactions may occur depending on variations in the virulence of the organism and the resistance of the host. Thus, in order to understand and appreciate the different infection phenomena, it is essential to have some knowledge of the various factors influencing the activity of the bacterium and the resistance offered by both the community as a whole and its individual members. Factors controlling the activity of the bacteria are numerous and varied ; questions of virulence, toxicity, number of organisms and their portal of entry are all involved.

The Virulence of Bacteria. The capacity of an organism to multiply in the tissues of the host and produce a severe form of infection is termed *virulence*. The term *invasiveness* is sometimes erroneously used as synonymous with virulence ; it, however, applies strictly to the power of the organism to invade the tissues of the host. Not only does virulence vary in the different bacterial species, but a marked variation may also be found in the individual strains of a given species. The species variation can be illustrated without difficulty ; infection with the *Staphylococcus pyogenes* usually gives rise to a localized purulent lesion, whereas infection with the *Streptococcus hæmolyticus* frequently produces a spreading lesion with subsequent invasion of the blood-stream. The variation in virulence shown by individual bacteria is indicated by the behaviour of a freshly isolated strain of the pneumococcus as compared with one kept in artificial cultivation for some time. The freshly isolated strain on intraperitoneal injection into a mouse leads to early death, whereas a similar injection of the old strain frequently does not manifestly affect the animal.

In some cases, particularly on the first isolation of a bacterium from the infected tissues, reproduction of the disease in the experimental animal is not readily obtained. By serial inoculation of the animals, however, the infectivity of the invading organism becomes greatly enhanced ; this is termed *exaltation* of virulence by animal *passage*. A striking and classical example of this is seen in the case of the rabies virus ; on fresh isolation the street virus reproduces the disease in rabbits only after a long incubation period, on serial intracerebral passage of the infected brain the incubation period decreases until the maximum virulence for the rabbit is reached ; the virus is then termed the "*fixed*" virus. While, however, the virulence is increased for the species of animal undergoing the passage, it is interesting to note that it may be lessened for other animal species ; *e.g.*, fixed virus is not so virulent for man as the street virus.

The opposite phenomenon, *i.e.*, a decrease in virulence, is termed *attenuation*. Attenuation of virulence may result in various ways : repeated subculture on artificial media, cultivation under unsatisfactory conditions, *i.e.*, using unsuitable media or by incubation at unfavourable temperatures. Pasteur, in the case of chicken cholera, found that, when the causative organisms were kept at room temperature for relatively long periods, the disease was not produced on inoculation of the cultures into susceptible fowls. Moreover, it was found on subsequent examination that the fowls injected with the avirulent strain were resistant to inoculation with freshly isolated virulent bacteria.

As a result of repeated cultivation on artificial media not only may the bacterium lose its virulence, but there is frequently an associated change in its cultural characters. The colonies on solid media become rough and dry, while the growth in broth is less marked and occurs in the form of a granular deposit. When these changes are associated with loss of virulence, the bacterium is termed *rough* in contradistinction to the *smooth* ordinary form. This variation from *smooth* to *rough* is usually styled : "S" → "R." Roughness of pneumococci is related to the loss of the *specific soluble substance*, or "S.S.S.," which is a complex carbohydrate associated with the capsule. In the case of the anthrax bacillus and the hæmolytic streptococcus, the normal virulent form is "roughish" in appearance. This is due to the physical arrangement of the bacteria, and is, thus, an entirely different phenomenon from the rough change described above.

While there is much to support the hypothesis that bacterial variation, particularly with regard to virulence and infectivity, is an important factor in determining the fluctuations in the prevalence of epidemics, direct and conclusive evidence of this relationship has not yet been produced. So-called "epidemic" strains of particular bacteria, which possess a high virulence and a high infectivity, have been postulated by different workers. It is also considered highly probable that a variation in the invading organism from the virulent form to a relatively avirulent form takes place in the tissues of the host towards the close of an epidemic ; this change occurs mainly in the tissues of hosts who have acquired some immunity. These are questions of great epidemiological and immunological importance, and they well deserve the close attention they are now receiving.

Number of Bacteria. There is no doubt that the number of bacteria implanted in the tissues plays an important part in the production of an infection. This point can be readily demonstrated in experimental animals by injecting serial dilutions of the bacterium. The highest dilution resulting in death is termed the *minimal lethal dose*, or M.L.D., and that causing definite infection the *minimal infecting dose*, or M.I.D. These terms were used extensively in the early days of bacteriology, particularly in the titration of antisera. Their use has, however, been subjected to much justified criticism, and their significance is now open to question. In view of the considerable number of variable factors involved in such tests it is absolutely impossible to obtain a standard fixed M.L.D. for a bacterium. Such a figure would obviously only hold for the actual experiment in which the particular organism was employed and tested. Consequently, instead of determining the M.L.D. a number of tests are made and the average lethal dose estimated ; this figure also has a limited value and applies strictly only at the time of the experiment.

The number of bacteria required to produce infection varies considerably with the different species. In the case of the anthrax and plague bacilli it appears that infection may result from invasion by a few bacteria, while with the weakly pathogenic species a considerable number of organisms is required to produce even a slight lesion. The number of bacteria is correlated with the virulence of the organism in the production of infection ; the more virulent the organism, the smaller the number required.

Route of Infection. The site of penetration and invasion by bacteria frequently plays an important *rôle* in determining the nature and extent of the infection. Cholera vibrios, when taken by the mouth in small numbers, may give rise to cholera ; in contrast the experiments of Ferran in Spain have shown that the subcutaneous inoculation of the living vibrios in relatively large doses does not produce any intestinal symptoms. Typhoid and dysentery bacilli also must be ingested to produce their respective diseases. The gonococcus gives rise to lesions of the genito-urinary tract and the eye. In contrast to this is the widespread distribution of lesions in syphilis and tuberculosis, the ætiological agents of which can produce lesions in most tissues.

The site of invasion by the anthrax bacillus considerably modifies the resultant infection. Infection through the skin gives rise to the localized malignant pustule, whereas infection *viâ* the respiratory tract produces a fulminating broncho-pneumonia. A similar state of affairs is found in the case of the plague bacillus ; infection through the skin results in bubonic plague, whereas infection *viâ* the respiratory tract leads to pneumonic plague.

The intact skin offers an almost complete protection against bacterial invasion. Bacteria are usually introduced as a result of some breach of its continuity, such as lacerations, punctures and incisions. Some organisms, such as the anthrax bacillus and *L. icterohæmorrhagiæ*, are, however, able to penetrate the undamaged skin or else enter through extremely minute abrasions. The intact mucous membrane is also a definite barrier against certain but not all bacteria. The coliform bacteria and the *clostridia* are normally present in the intestinal flora without causing any inconvenience, but on entrance into the tissues either through the damaged mucosa or by way of traumatic lesions infection frequently results. In contradistinction to this it is recognized that the uninjured intestinal mucous membrane is apparently penetrated easily by the intestinal pathogens, such as the cholera vibrio, typhoid and tubercle bacilli. The nasopharyngeal mucosa of susceptible individuals likewise offers little resistance to the diphtheria bacillus and certain strains of the hæmolytic streptococcus.

Toxic Substances produced by Bacteria. The injurious effect of bacteria on the body-tissues is of a chemical nature and is mainly, if not entirely, due to the action of toxic substances produced by the bacteria either during growth or on their disintegration after death. It has been mentioned that most bacteria produce active enzymes during growth and that these enzymes are able to break down carbohydrates, proteins and, to a less extent, fats. It would appear that these substances in themselves are not primarily responsible for the damage to the tissues, as many non-pathogenic bacteria also exhibit marked biochemical activity ; they may, however, participate in the secondary changes at the site of the primary infection. In the early days of bacteriology the bacterial poisons were thought to be *ptomaines*, and bacterial food-poisoning was termed *ptomaine poisoning.* It has, however, been found that ptomaines are merely cleavage products of proteins and constitute some of the end-products of bacterial metabolism. These substances are therefore not specific bacterial products and they play little part in the infective process. A further point of interest is that ptomaines are produced by relatively few pathogenic bacteria.

Our present conception of bacterial toxins originated in the work of Roux and Yersin, 1888–90, on the toxicity of the diphtheria bacillus. These workers found that the presence of the bacilli in the inoculum was not essential for the production of the characteristic lesions in the guinea-pig. The filtrate of a broth culture after passage through a bacterial filter had the same action as the whole culture ; the toxic action was thus solely due to some filtrable substance. This was termed an *exotoxin* and was considered to be a soluble substance secreted by the bacteria. Exotoxins were originally found to be formed by only a few bacteria, such as *C. diphtheriæ*, *Cl. tetani* and *Cl. botulinum*. In the case of other bacteria, such as the meningococcus and *V. choleræ*, it was found that the toxic substance was difficult to obtain and demonstrate ; it was frequently only liberated after death and disintegration of the bacteria had occurred. It was consequently thought that the toxin was intimately bound up in the bacterial protoplasm and was therefore quite different from the soluble exotoxin. This substance was consequently termed an *endotoxin* to indicate its close relationship to the body of the bacteria. These views were upheld until recent years, when a reconsideration of the question indicated that the separation of bacterial toxins into exo- and endotoxins was not such a simple matter. One of the chief difficulties in establishing the identity of the bacterial toxins has been the inability to determine their precise chemical nature. The only definite method available for recognizing their presence is by their action on the tissues of susceptible animals ; this may be an obviously injurious effect with, or without, a related antigenic action.

There appears to be little direct evidence that exo- and endotoxins are essentially different in origin. Early workers considered that exotoxins were secreted by the living bacteria. This does not appear to be probable, at least in the case of the diphtheria toxin, which is obtained in maximum concentration after growth for 7–10 days ; after incubation for this time a considerable number of the bacteria are dead and disintegration of the bacterial protoplasm is well advanced. In the case of a much less active toxin, that of *Cl. welchii*, maximum concentration is, however, reached during the active growth phase. An important factor in determining the activity of a toxin is undoubtedly the ability to obtain susceptible laboratory animals. Thus while lesions are produced in most laboratory animals by the diphtheria toxin, only the goat and the rabbit appear to be susceptible to the erythrogenic toxin produced by *Strept. hæmolyticus*, and even in these animals the action is not marked. The terms exotoxin and endotoxin are still widely employed, but it must be emphasized that there is no sharp line of demarcation between them. The application of the terms is more a question of usage and convenience than of accuracy of definition. The typical members of these two arbitrary classes differ considerably in a number of respects :

The *exotoxins* are antigenic, active in very small doses, relatively thermolabile, readily separated from the bacterial cell by filtration and exhibit definite tissue affinities. Examples fulfilling all these criteria are the toxins formed by the diphtheria and tetanus bacilli. Their potency is extremely high, minute doses, as little as 0·0005 c.c., of an active tetanus toxin being sufficient to kill a guinea-pig.

The *endotoxins* are non- or only slightly antigenic, active only in large doses, relatively thermostable, exhibit no definite tissue affinities and cannot be readily separated from the bacterial cell by filtration. Typical examples are the toxins formed by the meningococcus, gonococcus and the cholera vibrio.

Some toxins possess many, but not all, of the properties of the exotoxins ; *e.g.*, the botulinum toxin is relatively thermostable, the welchii toxin has a relatively large M.L.D., and the erythrogenic toxin of the streptococcus is not only relatively thermostable, but also has a high M.L.D., 5–10 c.c.

The most characteristic distinctions between exo- and endotoxins are thus based on (1) filterability, (2) characteristic tissue affinity, and (3) antitoxigenicity, *i.e.*, the ability to provoke or stimulate the formation of antitoxins on inoculation into the animal tissues. As examples of the marked tissue affinity exhibited by the various exotoxins, we find that the tetanus toxin attacks the anterior horn cells and red blood corpuscles, the shiga dysentery toxin the intestinal mucosa, the streptococcal toxin has an erythrogenic action and the diphtheria toxin attacks the heart muscle, kidneys and nervous tissue.

Bail, 1900–05, made a series of observations which indicated that, with certain bacteria, the pathogenic activity was increased by the addition of substances produced by the homologous bacteria in the animal tissues but not in culture. To these substances Bail gave the name *aggressins ;* he found that alone they were relatively non-toxic, their main action being to overcome the local tissue resistance and allow the bacteria to multiply and so produce their injurious action. This theory has been subjected to much criticism. The so-called " aggressins " have never been isolated, and as it has been proved that the addition of bacterial toxins to living bacteria increases their activity, it is probable that the aggressins do not constitute a separate entity but merely represent an action of bacterial toxins and somatic antigens.

A prominent feature of some infections is the local degeneration and disintegration of the leucocytes ; this was considered to be due to the action of a specific substance termed a *leucocidin.* In the light of our present knowledge it appears highly probable that this also is another manifestation of the activity of the bacterial toxins (cf., staphylococcus toxin).

There is no doubt that, in the case of some bacteria, there is a direct relationship between virulence and the presence in the bacterial cell of a special type of somatic antigen, which in the case of the pneumococcus has been termed *specific soluble substance.* This is a complex carbohydrate and virulent pneumococci have an appreciable amount, which is contained in the capsule and is specific for each type. The *rôle* played by this substance in the body is not fully understood, but some workers consider that it is responsible for the toxic symptoms of pneumonia ; there is no question that it generalizes, as it can be readily demonstrated in the blood-stream and urine in some cases of pneumonia. It appears to take up the antibodies for the homologous bacterial strain, thereby impeding phagocytosis, and probably interferes in other ways with the efficiency of the defence mechanism of the body. It thus possesses some of the properties of the so-called "aggressins." The virulence of *Bact. typhosum* is related to a somatic antigenic designated the "virulence" or Vi-antigen.

It must be recognized that, while the main or only offensive weapon of some organisms is the powerful exotoxin, *e.g.*, *Cl. tetani*, other organisms may produce several toxic substances, each of which may be concerned with the pathogenic activities. Thus *Strept. hæmolyticus* may form (1) an erythogenic toxin, (2) leucocidin, (3) hæmolysin, (4) fibrinolysin, as well as hyaluronidase, an enzyme facilitating diffusion in the tissues. The varied clinical picture of streptococcal infections is due to the irregular production of these substances by different strains and also to differences in the resistance of the host.

It is important to note that toxins are not produced solely by bacteria. Toxic substances, with properties similar to the bacterial toxins, can be readily obtained from castor-oil seeds, snake and scorpion venoms. They are all powerful poisons having a marked injurious action on the human tissues. This fact provides additional evidence of the chemical nature of bacterial activity.

Signs of Infection. Infection may be highly specific, *i.e.*, separate bacterial species produce their own distinctive lesions.

Thus the tubercle bacillus gives rise to the various forms of tuberculosis and never to diphtheria or anthrax; the typhoid bacillus produces enteric fever and not influenza. Pyogenic organisms, however, tend to produce a similar type of lesion in many tissues; thus purulent meningitis, pneumonia and empyema may be produced by a number of different organisms. It is also important to appreciate that, even with a given bacterium, the resulting infection may not invariably present the same features. This depends not only on the portal of entry, *e.g.*, anthrax and plague bacilli, but also on the balance of various controlling factors, such as the virulence and number of the invading organisms on one side and the resistance offered on the other, the resulting infection being subclinical, atypical or typical.

After the entrance of bacteria into the tissues signs of infection do not appear immediately. A varying period of time elapses before symptoms are observed; this is termed the *incubation period.* During the stage of incubation the bacteria first overcome the natural resistance offered by the tissues, after which they multiply and elaborate their offensive weapons. The duration of this period depends on many factors, including number and virulence of the bacteria, the site of infection and the resistance offered by the tissues. The local resistance may be considerably influenced by the mode of infection. When much trauma is applied during the introduction of bacteria into the tissues many of the local tissue-cells are destroyed; this obviously lowers the local resistance to a marked degree. The introduction of foreign matter with local tissue damage is largely responsible for the development of gas gangrene after street accidents or during war time, when wounds are frequently severe and grossly contaminated.

The changes following bacterial infection may occur locally at the site of infection or at a distance as a result of generalization of either the bacteria or their toxins. The nature of the local lesion depends largely on the nature of the invading bacterium. Many, *e.g.*, the streptococcus, staphylococcus and gonococcus, give rise to an acute inflammatory reaction characterized by swelling, redness and a marked exudation of polymorphonuclear leucocytes, which, with the broken-down tissue, form *pus.* This constitutes a suppurative lesion and the causative bacteria are said to be *pyogenic.* Bacteria are not, however, essential for the production of this type of lesion, the application of irritant chemicals, such as turpentine, to the skin or into the pleural cavity may also give rise to a marked exudation of polymorphonuclear leucocytes. Certain substances, including bacteria, have the property of attracting or repelling leucocytes; these phenomena are termed respectively *positive and negative chemotaxis;* the explanation of these reactions is not understood.

The diphtheria bacillus causes the production of much fibrin, which with the cellular exudate tends to give the lesion its membranous appearance. The tubercle bacillus and the *Trep. pallidum*, the ætiological agent of syphilis, cause a chronic granulomatous lesion, in which there is much fibrous tissue and a predominance of mononuclear cells. Certain anaerobes give rise to local tissue necrosis, often with the production of gas, as seen in gas gangrene.

In many instances the local tissue reaction, in which the cellular exudate plays a prominent part, is sufficient to keep the infection under control; in such cases the signs of infection remain localized. In the case of bacteria forming a powerful toxin a more generalized effect occurs; the toxin is produced locally, enters the blood-stream, giving rise to a state of *toxæmia*, then passes to and attacks susceptible tissue-cells. Thus lesions may arise some distance from the site of the bacterial invasion. When, however, the local reaction is unable to limit the spread of infection and dissemination of the bacteria takes place, the spread may take place in several ways :—

(1) By direct continuity along mucosa, epithelium, across adjacent surfaces or by natural channels as the ureters; examples are numerous—a spreading cellulitis, broncho-pneumonia following lesions of the upper respiratory tract, etc.

(2) By spread along the lymphatics to the regional lymph glands. An illustration of this mode of spread is seen in the case of bubonic plague. The plague bacilli penetrate the skin through minute abrasions and, without giving rise to any local lesion, pass to the regional lymph glands and form the characteristic buboes.

(3) By entrance into the blood-stream, giving rise to either septicæmia or bacteræmia and subsequent invasion of susceptible tissues. The invasion and persistence of bacteria in the blood-stream is generally termed *septicæmia* and is a common event in plague and streptococcal infections; in *bacteræmia* the presence of the bacteria in the blood-stream is considered to be transient and passive. Bacteræmia is present during the acute stages of many infections; *e.g.*, in the early stages of pneumonia pneu-

mococci are frequently present in blood-stream. In pyogenic infections a suppurative phlebitis with thrombosis may develop in one of the local vessels ; on the breaking down of the thrombus infective particles are disseminated in the blood-stream and multiple abscesses form in different tissues ; this condition is termed *pyæmia*.

Syphilis provides an excellent illustration of the various modes of spread of infection. After local implantation of *Trep. pallidum*, with later the production of the primary lesion or chancre, the spirochætes pass to the regional lymph glands causing an adenitis, after which the secondary stage with generalization by the lymphatics and blood-stream supervenes ; some time later, after a prolonged latent period, the chronic granulomatous tertiary lesions develop in various tissues.

In addition to the local symptoms directly arising from bacterial activity, evidence of general metabolic disturbance is usually present. The important signs are elevation of the body temperature, usually termed fever, general malaise and headache. An increase in the leucocyte count, or leucocytosis, is also often found ; in other instances, as during influenza, the leucocytes may be decreased, giving rise to a condition of leucopenia ; this event is, however, unusual. When toxæmia is marked there is usually an associated subnormal temperature and collapse.

CHAPTER IX

IMMUNITY

The resistance offered by the host to an infecting organism constitutes a complex subject to which the term *immunity* is generally applied. An individual offering resistance to infection is said to possess immunity; the converse to this "immune" state is susceptibility. There is, however, no sharp line of differentiation between immunity and susceptibility, the terms being purely relative. An individual may vary considerably in his response to infection with a given organism; thus he may be immune to a low-grade infection and yet susceptible to one of a similar but more intense nature. It has already been noted that the virulence of the invading organism and the resistance offered by the body-tissues are both factors of paramount importance in determining the outcome of a given infection, and it is now necessary to examine the second factor in more detail.

The study of immunity has usually, and perhaps advantageously, been carried out by considering the subject in two main sections, which are sub-divided according to the following scheme:—

- (1) Natural Immunity
  - species
  - racial
  - individual
- (2) Acquired Immunity
  - active
    - natural
    - artificial
  - passive
    - natural
    - artificial

1. Natural Immunity is the term employed to indicate the resistance offered by the body under normal conditions, *i.e.*, without any external stimulation by previous infection. The surface of the body is always in close association with a considerable number of bacteria, many of which, however, are harmless saprophytes. There is, nevertheless, a continual struggle for supremacy between the body and the bacteria and, in view of the relative infrequency of infection, it appears that the body normally offers a high resistance to bacterial invasion.

Important barriers to invasion by bacteria are undoubtedly the intact skin and mucous membranes. The skin is more or less impervious to most bacteria, but it is important to note that the anthrax bacillus and the yellow fever virus can pass through extremely minute abrasions as infections have resulted from the handling of infected organs at autopsies. In other cases definite breaches in the continuity of the skin are required to enable the organisms to gain entrance to the tissues ; illustrations of this are frequently encountered—streptococcal infections and tetanus following wounds and splinters, while gas gangrene may follow extensive traumatic lesions. The organisms responsible for these infections and many others are frequently present on the intact skin or in dirt and soil, and are able to produce infection when the barrier presented by the skin is broken down by some injury, during which they are introduced into the tissues. The secretions of the skin, such as the sweat, assist in resisting infection by their acidity and cleansing action, by which they tend to eliminate bacteria. When, however, the sweat glands are blocked and the secretion becomes stagnant, infection readily occurs and local pustules or boils develop.

The mucous membrane does not offer the same resistance to bacteria as the skin. The presence of hæmolytic streptococci or diphtheria bacilli in the nasopharynx frequently leads to infection, while syphilis and gonorrhœa may follow the implantation of their respective agents on the apparently intact urethral mucous membrane. The intestinal mucous membrane is, however, relatively impervious to the coliform and anaerobic bacteria, but appears to be readily penetrated by the cholera vibrio and the Salmonella and dysentery organisms.

The various secretions produced by the cells in the different mucous membranes may possess a marked bactericidal activity. The gastric juice, mainly in virtue of its acidity, is particularly active ; many bacteria are introduced and ingested during eating and drinking, but most of these are destroyed in the stomach. Some may, however, escape the acid, being protected by the food particles or by rapid passage through the stomach, and others may be resistant to the acid. The gastric juice acts also in the upper portion of the duodenum, which normally contains few bacteria. The acidity of the vaginal secretion is a particularly important factor in determining the vaginal flora, aciduric bacteria, such as Doderlein's bacillus, being normally predominant.

A bactericidal substance, *lysozyme*, has been obtained from the nasal secretion, skin tissues and tears. While lysozyme has a marked injurious action on a certain bacterium, *M. lysodeikticus*, its effect on more parasitic bacteria is uncertain. While there is little doubt that it participates in the body defence, its *rôle* does not appear to be of any great importance.

Certain non-specific antibacterial substances, termed *β lysins*, *leukins* (derived from leucocytes) and *plakins* (derived from platelets), are commonly present in serum and are considered to play some part in resistance to infection, but these are of little practical importance.

These general factors are concerned in the resistance naturally offered to infection. The various animal species are not always equally susceptible to infection, and other factors are undoubtedly involved. These will be considered under the titles of species, racial and individual immunity.

Species Immunity. Certain diseases, *e.g.*, tuberculosis, anthrax, psittacosis and rabies, occur spontaneously in both man and animals. There are, however, many human diseases which do not occur spontaneously in animals ; thus syphilis, measles, meningococcal meningitis, poliomyelitis, leprosy, etc., are essentially diseases of man. In some cases, *e.g.*, syphilis and measles, the disease may be reproduced experimentally in certain animals by the injection of large doses of the virulent organism, but the range of susceptible animals is often limited and the resulting infection may be relatively slight. There are also many diseases of animals, *e.g.*, rinderpest, Johne's disease and distemper, which are not encountered in man. The explanation of this absolute insusceptibility is unknown, but there is no doubt that it is mainly due to variations in the receptivity of the tissues of the different species. Metabolic activity varies appreciably in different animals and a profound biochemical difference must exist in the various tissues ; these changes probably govern the ability of bacteria to multiply in the different tissues. Bactericidal substances are not usually present in the sera of naturally refractory animals, so that participation of this factor can be excluded in most cases.

Cold-blooded animals are not generally susceptible to diseases of warm-blooded species ; it is, however, possible in certain instances to overcome this insusceptibility by artificially raising the body temperature of the cold-blooded species. A similar trend of events is seen in the case of birds, which normally possess a relatively high temperature ; by lowering this artificially the birds may lose their resistance to several human and animal pathogens.

Racial Immunity. Differences in response to various infections are sometimes encountered in the various races of the same species, but in few instances only are these true examples of natural immunity. Thus inhabitants of tropical regions are generally more resistant to yellow fever than people from other regions. This has been found to be merely an instance of acquired immunity resulting from some slight or subclinical infection during childhood and not to any essential genetic peculiarity. Coloured races are usually more susceptible to tuberculosis than white people ; the probable explanation is, however, that, in view of the natural distribution of the disease, resistance due to previous infection is more likely in white than in coloured people. Similar variations in susceptibility can be found in animal diseases, as an example distemper in dogs may be cited, one of the difficulties in the research work on this disease was that some dogs were resistant to infection due to previous subclinical infection. This difficulty was only overcome by breeding dogs under strictly isolated conditions. On the other hand a possible example of a racial natural insusceptibility to infection in animals is found in Algerian sheep, which are considered to be more resistant to anthrax than other types of sheep. In all instances, however, the differences in susceptibility are only relative ; the infection can usually be induced in the resistant members by the use of a sufficiently large dose of the responsible organism.

Individual Immunity. Individuals in a community vary considerably in their response to invasion by a pathogenic bacterium. All types of the disease may be encountered—typical, atypical and subclinical infection, while some may not be infected. This variation of response in an ordinary community is usually due to the presence of some previously acquired immunity, but, in the case of a virgin population, other factors are largely responsible. Examples of the former class are seen in the resistance of adults to the acute infectious diseases of childhood, due in the majority of cases to previous infection. Some workers have suggested that the difference in response of adults and children to these infections is the result of a physiological change of the respective tissues. They consider that with increasing years the tissues become more resistant by a physiological process of maturation. The evidence supporting this theory is, however, far from conclusive and consequently its acceptance is not justified.

The main non-specific factors influencing the course of infection are the state of the general health and the presence of any predisposing conditions. These, however, apply equally in considering both natural and acquired immunity. The influence exercised by the general condition of the individual is well known. People in good health, well-nourished and in good surroundings are definitely more resistant than those who are weakly developed and underfed. Alcoholism tends to lower the general resistance, as illustrated by the incidence and severity of pneumonia in alcoholics; fatigue, particularly if prolonged, has a similar effect. The presence of some predisposing factor also exerts a definite influence on the incidence and localization of infection. This is seen in infections following traumatic lesions; osteomyelitis not infrequently follows a blow on a limb, and tuberculosis is very common in individuals employed in silica works. These outside factors play an important *rôle* in determining the site of infection, as they lower the local resistance of the tissues and create an increased permeability of the vessels, both of which tend to predispose to infection. Deficiency of vitamins in the diet also tends to lower the resistance of the body; this is particularly found in the case of vitamin A, which is considered to be anti-infective, and also perhaps with vitamin C. The exact nature of action is, however, not understood, but it is probably related to the development of local tissue changes, by which the resistance to bacterial invasion is decreased.

2. Acquired Immunity may be either active, in that the increased resistance is due to some reaction of the person's own tissues, or passive, in that the immunity is obtained by the introduction from without of immune bodies induced in some other person or animal.

Active Acquired Immunity. Active immunity may arise naturally or be induced by artificial stimulation. In each form the reaction is essentially the same, as it is a response of the person's own tissues to the introduction of a foreign substance. In artificially inducing immunity an attempt is made to increase the resistance against subsequent infection with the minimum risk and discomfort. This is consequently a question of great importance in preventive medicine.

Natural active immunity is exemplified by those cases in which a single attack of the spontaneous disease confers an increased resistance against a second infection. In many instances a high degree of immunity follows an infection, as in enteric fever, diphtheria, measles, plague, scarlet fever, etc. In many cases subclinical infection also plays an important *rôle* in the production of immunity ; for example, the frequent resistance of adults to many diseases, in the absence of a previous attack, is considered to be the result of subclinical infection during childhood. While this immunity is definite and of a high grade, it is not correct to term it " absolute " or " permanent." In exceptional circumstances, as a result of either a decrease in the general resistance or an overwhelming infection, the defence mechanism may be broken down and a second attack ensue. In other cases a low grade of immunity, which is often transitory, may follow an infection, as in influenza and the common cold. It is possible that this absence of immunity may be due to the presence of immunologically distinct types of an organism and thus be more apparent than real, *e.g.*, pneumococcal pneumonia may be produced by many immunologically different types of the organism and an increased resistance against one does not protect against the other types ; in foot and mouth disease of cattle there are at least three distinct types of the virus, which give no reciprocal immunity.

Artificial Immunization. It has long been recognized that the immunity following an attack of many infectious diseases is sufficient to prevent a second attack and repeated attempts to increase the resistance by the artificial induction of a modified form of the disease have consequently been made. In the East immunization against small-pox by the inoculation of portions of the small-pox pustules has been practised since the eleventh century. Artificial immunization, however, first became a weapon of general application against acute infections as a result of the work of Jenner during the late eighteenth and early nineteenth centuries. Jenner investigated and confirmed the dairy-workers' contention that persons who had been infected with cow- or horse-pox subsequently escaped infection during the severe epidemics of small-pox. As a result he introduced the preventive measure, now universally known as "*vaccination*", in which the resistance to small-pox is increased by the cutaneous application by scarification of the cow-pox, or vaccinia, virus. Further progress in artificial immunization was delayed until the work of Pasteur, who observed accidentally, during an investigation of fowl-cholera, that the injection of an attenuated culture was able to protect fowls against a subsequent inoculation of the virulent organism. Pasteur devoted much time to the question of artificial immunization and later introduced his classical methods for inducing protection against anthrax and rabies by means of the modified and attenuated organisms.

These observations opened a new field of research which received much attention from workers in many countries. Considerable progress was made and Pasteur's observations were confirmed and elaborated. Another striking advance was the conclusive demonstration by Wright, during the campaign in South Africa at the end of the nineteenth century, of the value of killed organisms in affording protection against enteric fever. These successful results popularized the use of killed bacteria or " vaccines " as a prophylactic and therapeutic measure ; vaccines were subsequently prepared by numerous methods and extravagant claims of satisfactory results in preventing or clearing up bacterial infections have since been made. Many of these claims have not been substantiated, but there is no doubt that vaccines, when properly controlled, may be particularly useful prophylactic agents.

Another important advance in immunization was the discovery that, on the inoculation of horses with potent diphtheria and tetanus toxins, antitoxins were induced in considerable amounts. The subsequent use of the sera of these animals for therapeutic purposes yielded striking results, particularly in the case of diphtheria in which the severity and duration were both definitely reduced. The use of toxins as a means of actively increasing the resistance of man was not at first very successful owing to the marked reactions which followed the injection into the tissues of even very small doses. In recent years further progress was made by Ramon when he found that, on treating toxins with formalin for variable periods of time, they lost their toxicity but retained their antigenicity. This modified toxin, which has been termed *toxoid* or *anatoxine*, has proved of great value not only as a prophylactic measure in some human diseases, but also in immunizing horses for the preparation of antitoxic sera.

The number and variety of preparations available and in use at the present time for both therapeutic and prophylactic purposes are considerable. In many cases there is, however, little evidence to justify their use. The evaluation of vaccines is a complex problem ; a major difficulty is that the determination of their efficacy in increasing the resistance of an individual against infection with the homologous organism cannot be performed with any degree of accuracy. The following three methods are in use for assessing the value of these agents, but their application is somewhat limited :—

(1) This consists of a statistical survey of field experiments. Individuals liable to be exposed to the same risk of infection are divided into two groups, one of which is inoculated with the agent and the other not. The subsequent incidence of spontaneous infection in the two groups is determined and compared. This procedure, when applicable, frequently gives the most valuable and reliable results, but the interpretation of the results requires much caution ; it is advisable to co-operate with a statistician.

(2) Another method is the inoculation of the preparation into susceptible animals, which are tested some time later by the injection of virulent organisms ; the development or not of the characteristic infection after the second inoculation is indicative of the degree of resistance induced. This is an exacting test, which can only be made in experimental animals, and in many instances the pathogenesis of the experimental disease is different from that of the spontaneous disease in man. The two conditions are thus not strictly comparable and the results are only of relative value.

(3) The examination of the serum for the presence of antibodies after the inoculation of the vaccine often provides useful information of the body response. This method, however, gives an indication of reaction to the inoculum rather than an accurate assay of induced resistance.

In view of the uncertainty of evaluating these agents it is not surprising that many varieties are available. The most important preparations in use at the present time are given below.

(*a*) **Living Attenuated Organisms.** The use of living organisms is limited as, in all cases, it is essential that any risk of inducing the disease itself must be completely eliminated. It has been found possible in some instances to obviate any such risk by adapting the organism to the tissues of a different animal species or by growing it under unfavourable environmental conditions, *e.g.*, at temperatures above that optimal for growth, as in the preparation of the anthrax vaccine which contains the bacilli grown at 42°–43° C. Vaccination with the vaccinia virus, which is a modified form of the variola virus, is an example of the value of a modified living organism ; also, in the prophylaxis of rabies, the living virus, modified by intracerebral passage in the rabbit, has been employed with great success. During the last decade Calmette has advocated the use of living attenuated tubercle bacilli as a preventive measure against tuberculosis in infants. Great claims have been made for this vaccine, which is termed " B.C.G. " (Bacille-Calmette-Guérin), and it has been widely used on the Continent. Attenuated organisms are also used with varying degrees of success in combating some animal diseases, such as anthrax and rinderpest. The yellow fever vaccine is a further example of a modified virus.

(*b*) **Killed Organisms.** Owing to the limited application of living vaccines, organisms, killed in various ways, are mainly employed for inoculation into man. Diverse agents have been used to kill the bacteria ; heat at 56° C., phenol, alcohol and formalin are those usually employed in practice. It is necessary in all cases to consider the antigenic structure of the organism before deciding the method of preparing a vaccine, as it is essential that the effective antigens should not be destroyed ; T.A.B. vaccines are now treated with alcohol in order to preserve the labile " Vi " factor. While there is some doubt whether dead organisms are as effective as living attenuated forms, their value in increasing the resistance to certain infections is beyond dispute. Their use as a prophylactic measure against enteric fever, cholera and staphylococcal infections, to quote only a few examples, has yielded very satisfactory results. The main advantages of " killed" vaccines are that the risk of inducing infection is eliminated and the reactions following the inoculation are relatively slight.

(*c*) **Bacterial Extracts or Filtrates.** Various types of bacterial extracts have been tried from time to time, but the most satisfactory results have been obtained from bacterial filtrates containing the powerful toxins. These toxins, formalized to form toxoids, are now employed extensively both for prophylaxis against diphtheria and tetanus and for the preparation of antitoxic sera in horses. A recent development has been the extraction and purification of active somatic antigens for prophylactic immunization, *e.g.*, " O " somatic antigen of *Bact. dysenteriæ* and the complex polysaccharides of the pneumococcus. These have not yet been subjected to extensive field trials.

Many other preparations such as sensitized vaccines, lipovaccines and bilivaccines have been introduced by different workers, but they are not in general use and need not be considered here.

Passive Immunity. Passive immunity may also arise naturally or be artificially induced. It is important to note that passive immunity is short lived as compared with active immunity. In the absence of fresh stimuli antibodies, introduced from an outside source, tend to disappear from the serum after a short time, usually within a few weeks or months.

Natural Passive Immunity is dependent on the transplacental passage of immune bodies from the mother to the child during intra-uterine life or later by the milk during early infancy. The immunity so obtained is only transitory, as the immune bodies tend to disappear after a few months, and also it is usually of a low grade. Antiviral bodies in the case of poliomyelitis and yellow fever, and antitoxin in diphtheria, have been found in infants up to 6 months of age, but over this age their demonstration becomes increasingly difficult. Risk of infection at this age-period is generally not very high, but in the case of widespread epidemics the increased resistance may modify the disease process so that atypical or subclinical infections not infrequently occur. The occurrence of a subclinical or atypical infection in infants possessing a passive immunity, naturally acquired, is considered to be the factor responsible for the marked resistance subsequently exhibited by native people to the virus of yellow fever.

Artificial Passive Immunity is obtained by the introduction of serum or blood, containing antibodies, from an outside source. The sources from which this serum or blood is collected are either individuals, convalescent or recovered from the corresponding disease, or animals, which have been suitably immunized. Serum from convalescent or recovered cases is employed when a satisfactory animal cannot be found for immunization. The use of human serum is less convenient than that collected from immune animals ; for, while the antibody content of the two may be comparable, the quantity obtainable from one individual is much less than that from an animal such as the horse. Measles and poliomyelitis are both diseases in which human convalescent serum is employed.

The horse is the most satisfactory animal for the preparation of immune sera ; large quantities of blood can be withdrawn from the jugular vein at one bleeding and moreover repeated bleedings can be made without causing the animal any appreciable discomfort. These animals respond well to the inoculation of most antigens, and after a course of injections the serum usually contains a high concentration of antibodies. As some may fail to respond, sample bleedings are taken during the course of immunization to determine the antibody titre. When the use of horses is impracticable, sheep, goats and asses have been used. Before any animal is immunized to obtain serum for therapeutic or prophylactic purposes, it is essential that it should be in good condition and free from disease; to ensure this a veterinary examination is required before the immunization is commenced.

Immune sera may be divided into three classes:

(*a*) antitoxic,
(*b*) antibacterial,
(*c*) antiviral.

In each case the serum may be used either for therapeutic or curative purposes, *i.e.*, in the treatment of an established infection, or for prophylaxis, *i.e.*, as a preventive measure before infection has taken place or immediately after infection, but before the disease process has developed. When active measures are available for prophylaxis, as in diphtheria and scarlet fever, the application of serum should be restricted to urgent cases. The reasons for this are the relatively short duration of passive immunity and also the avoidance of inducing a hypersensitiveness to the serum.

Antitoxic sera, *i.e.*, those prepared by the injection of the modified exotoxins, have given the most dramatic results. The value of these sera is shown by the results following their application in the therapy in diphtheria, and in the prophylaxis of tetanus and gas gangrene. The severity of these conditions has frequently been reduced in a striking manner by administration of antiserum. The antitoxin acts directly on the free toxin, which is neutralized, but once the toxins have reached susceptible cells antitoxin is without effect; therefore in therapy antitoxic and indeed all other sera should be given at the earliest possible opportunity. A striking illustration is the marked difference in the value of antitoxic serum given prophylactically and therapeutically in tetanus. As a prophylactic measure it has proved highly successful, but in the treatment of the disease, *i.e.*, when the toxin has reached the susceptible nerve-cells, it is of very limited value.

Antibacterial sera have, for the most part, given disappointing results. This has probably been due to the failure in the past to appreciate the complex antigenic structure of many bacteria and the importance of the type-specific fraction in immunity reactions. Satisfactory sera are difficult to prepare, but in some instances, particularly pneumococcal pneumonia and meningococcal meningitis, they have proved useful therapeutic agents. In these conditions, however, serum therapy has been almost entirely replaced by sulphonamides or penicillin.

Antiviral sera have been employed extensively in recent years for both the therapy and prophylaxis of virus diseases, *e.g.*, measles, poliomyelitis, yellow fever, etc.; while there appears to be little doubt that in measles at least the results have been satisfactory, in many of the other conditions the results have been less convincing. It is as yet impossible to give a definite opinion on the value of these sera.

It is important to note that all sera prepared for sale must, before issue, be adequately tested and titrated. This is a relatively easy matter in the case of the antitoxic sera, but antibacterial and antiviral sera are usually difficult to standardize. For many sera definite standards have been adopted and, in this country, the manufacture and sale of these sera is controlled by the Therapeutic Substances Regulations passed in 1931. This question has recently become less involved by the tendency to adopt the international standards laid down by the Permanent Commission of the Health Organization of the League of Nations. In this way the standards used in different countries become regular and thus more comparable.

Unpleasant reactions not infrequently accompany the introduction of foreign serum into the human tissues. As these are mainly caused by the non-specific proteins present in the serum, they may be obviated or minimized by separating and concentrating the antibody-containing fraction. This may be accomplished in many ways, of which the one most commonly used is the addition of appropriate strengths of ammonium sulphate and subsequent dialysis. In this way not only are the unpleasant reactions avoided, but the amount of antibody in a given quantity is greatly increased. The antitoxins are contained in the pseudoglobulin fraction and are consequently readily concentrated; antibacterial and antiviral bodies are associated with the so-called euglobulin fraction, which can only be separated with difficulty (cf. p. 112).

Herd Immunity. The reaction of a community or herd to infection is chiefly dependent on the distribution of the infecting organisms and the resistance offered by the herd as a whole.

When the general resistance is slight and the invading organism is sufficiently widespread and active an extensive outbreak or *epidemic* results. When, however, the general resistance is high cases occur sporadically and the disease is termed *endemic*. When extensive outbreaks of a disease are present in several countries at the same time, it is said to be *pandemic*, *e.g.*, influenza in 1918–19. An illustration of an epidemic is seen when such diseases as measles, the common cold and poliomyelitis are introduced into virgin populations, such as remote islands, and a widespread outbreak or epidemic involving individuals of all ages has resulted. In this country poliomyelitis is not common, sporadic cases only being encountered ; the disease is thus endemic. In contrast to this, large outbreaks or epidemics of measles, in which the cases are largely confined to children, occur with marked regularity.

An important factor in determining the incidence of a disease is naturally the distribution and persistence of the infecting organism. As a result of strict hygienic measures the cholera vibrio is mainly limited to tropical regions. Cholera outbreaks are consequently not encountered in this country, but they occur with great frequency in the East. When infection is dependent on some outside host, the exclusion of this will obviously reduce the incidence of the disease ; *e.g.*, the precautions taken in this country to exclude infected rats and rabid dogs have been responsible for the considerable decrease here of plague and rabies respectively. With the recent popularity of air transport the carriage of infected mosquitoes to uninfected regions and the consequent introduction of yellow fever has been the cause of some alarm and rigorous inspection of aeroplanes in these districts is enforced. Infection may also be conveyed by drinking water, milk and food ; these vehicles of infection can, however, be controlled by adequate inspection and treatment, such as the boiling of water or milk, the pasteurization of milk and the cooking of foods.

Whenever the exclusion or removal of the infective agent is impossible attention must be directed to the other important factor, *i.e.*, the resistance of the community. This should be increased by specific and non-specific means where possible. The non-specific means of increasing the resistance consist chiefly in directing attention to the hygienic surroundings, prevention of overcrowding, nutrition, etc. The resistance may be specifically augmented by various preparations. The practice of vaccination during infancy has greatly decreased the incidence and severity of small-pox; the inoculation of soldiers with the T.A.B. vaccine has had a similar effect on the enteric fevers, which had such a crippling effect during the South African War; immunization against diphtheria by means of the various toxoid preparations is now widely practised with good results; the use of tetanus toxoid, yellow fever vaccine and typhus vaccines was a striking success during the recent Great War.

The application of prophylactic measures is one of the major problems of preventive medicine and also one in which considerable advances have been made in recent years. The application of skin tests, such as the Schick and Dick tests in diphtheria and scarlet fever respectively, which differentiate immune members of a community from susceptibles, is particularly valuable in the selection of individuals suitable for immunization.

The questions which have just been considered indicate that the control of an infection in a community involves a consideration of two important factors. On the one hand there is the practicability of increasing the resistance of the herd by specific and non-specific measures, and on the other hand the possibility of excluding or eliminating the infecting agent, which involves, *inter alia*, the control of carriers. These constitute two of the major problems confronting preventive medicine, and while considerable progress has undoubtedly been made, there is still much to be done, especially in diseases propagated by aerial transmission.

Local Immunity. In the early days of bacteriology there was much controversy over the respective merits of the cellular and humoral responses as factors in producing resistance to infection. Both sides had staunch supporters, and considerable research was carried out to further the respective claims. As a result it was finally decided by all workers that both factors played important parts in the immunity response.

In recent years the question was raised again by Besredka who, in numerous papers, stated that in many intestinal and dermal infections there is a specific localization of the responsible bacteria in the particular tissues irrespective of their route of introduction into the body. Moreover, in resistant animals the local tissue-cells only are concerned with the resistance offered to the infection. He also claimed that the resistance of the intestinal cells can be increased by stimulation with special vaccines, *bilivaccines*, which are given by the mouth. The appearance and significance of antibodies were discounted in many of these observations. In the case of the skin it was claimed that the resistance of the cells can be increased by the application of broth filtrates of certain pathogenic organisms; sterile filtrates are on sale, under the name of *antivirus*, for use in the treatment of various skin lesions.

These views are, however, not generally accepted. It is considered by most bacteriologists that the production of a specific local immunity is merely a specialized manifestation of the general immunity response. In such cases protective antibodies can usually be demonstrated in the serum. In many instances it appears highly probable that the increase in resistance exhibited by the local area is mainly non-specific and is due to the inflammatory reaction induced by the initial local applications. The reactivity of the local tissue-cells is thereby increased and they consequently respond more readily to subsequent infection.

CHAPTER X

ANTIGENS, ANTIBODIES AND THEIR REACTIONS

There is little doubt that the increased resistance which is acquired after many bacterial infections is closely associated with the appearance of antibodies in the serum of the individual. These antibodies can be demonstrated by their action on the homologous bacteria, which may be clumped, lysed or more readily ingested by the body-cells according to the characters of the bacterial suspension and the conditions under which the reaction is carried out. The substances which stimulate the production of the antibodies are termed *antigens.*

The exact significance of antibodies in the resistance of the body to infection is not fully understood, although much work has been devoted to the subject and much controversy has arisen therefrom. During the last quarter of the nineteenth century and the early years of the twentieth century two important schools of thought existed, and the attempts to support the views of one or the other have added much to our knowledge of the subject. One school followed the teaching of Metchnikoff and considered that certain tissue-cells, which ingested the bacteria and were termed *phagocytes*, constituted the factor of paramount importance in resistance to infection; this was the *cellular* hypothesis. The other doctrine, which was supported by Ehrlich, postulated that the antibodies provided the main defence against bacterial invasion; this was the *humoral* hypothesis. The accumulation of information has, however, indicated that neither of the theories alone can explain all the phenomena encountered and it is now generally accepted that both cellular and humoral factors play important *rôles* in the defence mechanism of the body.

Antigens. An *antigen* may be defined as any substance that, when introduced in an unaltered form into the animal tissues, stimulates the production of substances or *antibodies*, which react specifically with the corresponding antigen.

Many substances, *e.g.*, bacteria, blood corpuscles, serum, bacterial and vegetable toxins, may act as antigens, and in all cases a marked degree of specificity is exhibited. This specificity is, however, dependent on chemical and not biological factors.

Moreover, one bacterial species may contain many different antigens and, on the other hand, varied species may contain an identical antigen. This interrelationship of antigens is frequently encountered in the different bacterial species and has added considerably to the complexity of the serological reactions.

While the exact chemical composition of antigens has never been determined, there is much evidence to indicate that complete antigens must contain protein. Proteins, in order to be antigenic, should be foreign to the animal injected, be soluble in the animal tissues, and possess a certain molecular size ; it has been found that proteins on hydrolysis tend to become non-antigenic. The factor to which proteins owe their antigenic capacity is not known, but as gelatin, which lacks aromatic radicals, is non-antigenic it has been suggested that the aromatic radicals constitute a factor essential for antigenicity. Evidence to support the chemical specificity of antigens is obtained from the fact that by altering the chemical structure of a protein molecule its specificity is also changed and different antibodies are produced on injection into the tissues. While substances other than proteins are not complete or true antigens, there is no doubt that carbohydrates and lipoids may have a partial action. It has been found in the case of the pneumococcus that the specificity of the various types is not due to the presence of different proteins in the bacterial cells, but to the presence of a specific complex carbohydrate in each type. These substances have been extracted from the cocci by precipitation with alcohol and have been found to act as partial antigens. They are unable to stimulate the production of antibodies when separated from the intact antigen, but can react *in vitro* with serum prepared against the homologous pneumococcus ; Landsteiner has termed these partial antigens "*haptenes*". It has, however, been recently found that the specific carbohydrates of Types 1 and 2 pneumococci may exhibit a certain degree of antigenicity. As a rule the presence of a conjugated protein fraction is necessary to give the specific polysaccharide fraction its antigenic properties ; this applies to many bacteria.

There is also no conclusive evidence that lipoids act as true antigens. They behave in a similar manner to the haptenes, *i.e.*, they do not stimulate antibody production, but can react *in vitro* with homologous antibodies. It is probable, however, that the addition of lipoids to proteins may alter the antigenicity of the latter.

Bacteria constitute complex antigens. As an illustration we find that the typhoid bacillus contains several main antigenic components, some of which are associated with the body and others with the flagella ; the structure of the pneumococcus consists of a non-specific protein and a type-specific carbohydrate ; the *Strept. hæmolyticus* (Group A strains) is composed of a type-specific protein, a non-specific nucleo-protein and a polysaccharide. These varied factors require close attention in the performance and interpretation of the serological reactions. It is important to note that antigenic factors concerned with type specificity and antibacterial immunity are situated on the surface of the bacterial body.

Antibodies. An *antibody* is any substance that appears in the blood of an animal as a result of the entrance into the tissues, *i.e.*, parenteral introduction, of an antigen, and that reacts specifically with the same antigen *in vitro* and *in vivo*. Antibodies have never been isolated in a pure state, as it is impossible to exclude the presence of a certain amount of the normal serum protein. Their presence is recognized by means of the reactions produced by them. They have consequently been designated by the reactions produced on mixture *in vitro* with the corresponding antigens : one giving a precipitate is termed a *precipitin :* an antibody causing clumping or agglutination of the bacteria is called an *agglutinin :* if lysis and disintegration of the bacteria follow the antibody is a *bacteriolysin :* a *bactericidin* kills bacteria without producing lysis ; if the bacteria become more susceptible to ingestion by the phagocytes the antibody is termed an *opsonin* or *bacteriotropin :* if the antibody neutralizes the toxin produced by a bacterium it is an *antitoxin.*

Although the precise nature of antibodies has not been determined, there is much evidence to indicate that they are closely associated with the serum globulins. By means of fractionation tests, *e.g.*, the addition of definite concentrations of ammonium sulphate to the serum, three fractions can be obtained—albumen, euglobulin and pseudoglobulin. Repeated observations have shown that, although the various antibodies are not restricted to one fraction, the major portion is constantly associated with a single element. Thus the antitoxin is precipitated almost entirely with the pseudoglobulin fraction, while the other antibodies are mainly associated with the euglobulin fraction. These facts are utilized in the preparation of sera for therapeutic purposes. The protein contained in serum may give rise to a marked reaction on inoculation into the human tissues. By separating the fraction containing the required antibody, not only is the antibody content increased or concentrated, but also a large amount of extraneous protein is eliminated. The risk of reaction is thus reduced and the value of the serum increased. Refined sera, prepared by treatment with proteolytic enzymes to digest extraneous proteins, are now available. On account of this, serum for specific therapy and prophylaxis is employed whenever possible in the concentrated form.

New methods for the fractionation of serum have recently been introduced by Cohn and his colleagues in America. These involve the use of alcohol fractionation, electrophoresis and the high-speed centrifuge. Albumen was found to be the principal fraction, containing some 60 per cent. of the total proteins ; the **gamma globulin** was found to constitute about 11 per cent. of the proteins and to contain most antibacterial immune bodies of the serum. The γ-globulins have not yet been subjected to extensive trials, but preliminary observations indicate that they modify an attack of measles. Antitoxins are associated with the ' Beta ' globulins.

Site and Mode of Antibody Formation. Although much work has been carried out to determine the site and manner of antibody formation, the responsible mechanisms are not definitely known. It is therefore not surprising that several theories have been postulated to explain the origin of antibodies.

At one time it was suggested that antibodies consisted of antigens in a modified form. Little evidence could be obtained to support this hypothesis, and it has now been abandoned. It is well known that in some instances the injection of even minute quantities of an antigen results in considerable antibody formation.

Ehrlich, in devising his side-chain hypothesis, postulated that antibodies were receptors shed from certain tissue-cells as a result of stimulation by the antigen. This theory was subsequently elaborated to satisfy the various antigen-antibody phenomena encountered by various workers, but definite evidence to substantiate it has never been obtained.

As it was known that the cells of the reticulo-endothelial system played an important part in the removal of foreign material from the blood-stream, the possibility that it might also be concerned with the formation of antibodies was investigated. The reticulo-endothelial system is widely distributed in the body ; it includes endothelial cells of the spleen, liver (Kupffer cells), lymph glands, bone marrow, and also the wandering macrophages of the tissues and the blood. The difficulties of establishing conclusively the participation of this system in the formation of antibodies are considerable, owing to its wide distribution in the body. Nevertheless, experiments carried out to date indicate in no uncertain manner that the R.E. system is involved in the formation of the antibodies ; its exact function in this respect, *i.e.*, the precise mechanism of antibody formation and the mode of action of the antigens, is, however, undecided. The experiments employed in the investigations have been concerned with the injection of antigens before and after both splenectomy and "blockade" of the R.E. system ; the serum is examined after a short interval for the presence of antibodies. Blockade of the R.E. system is performed by inoculating intravenously large quantities of some foreign colloidal material such as indian-ink ; the colloidal particles are taken up by the cells and cause a temporary inhibition of function. The results obtained by various workers have differed, but in many cases it has been shown that both splenectomy and blockading definitely interfered with the formation of antibodies.

Considerable variation is found in the antibody response following the inoculation of an antigen into an animal. This variation is dependent on several factors, particularly the amount and type of antigen injected, the animal employed and the route of inoculation.

As a general rule an animal will not produce antibodies against tissues of the same species ; *i.e.*, the antigen must be foreign to the species. Important exceptions are the antigenic constituents of human red cells, in particular the Rh factor (cf. p. 124). Some animals are more responsive than others ; antibody formation in the rabbit, for example, is considerably greater than in the guinea-pig. The amount of antigen injected also influences the antibody response ; a minimal amount is always required and above this antibody formation is augmented until a point is reached where larger doses do not result in increased antibody production. Antibody formation also depends to some extent on the concentration of the antigen in the blood-stream ; consequently the intravenous route is usually more effective than the intramuscular and subcutaneous routes. Following the initial injection of the antigen no response is detectable for several days. This represents the initial induction phase ; this is followed by the appearance in the blood-stream of the homologous antibodies, which soon reach a maximum titre; the titre then tends to fall, rapidly at first, but later more slowly until the antibodies finally disappear. This process may take several months or years. The disappearance of circulating antibodies does not necessarily indicate a complete return to the original normal state; a *basal* immunity usually remains. This is manifested in the response to a subsequent injection of the same antigen; the antibody response is both more rapid and more marked than that resulting from the primary stimulus, while the decrease in titre occurs more slowly.

It is interesting to note that after a primary stimulation of antibody production a response, frequently very slight, may be elicited by the injection of antigens which, while not identical are closely related to the original. This may be due either to the close similarity of the chemical structure of the different antigens or to a non-specific stimulation of the antibody-forming tissues. This is referred to as an *anamnestic reaction.*

Natural Antibodies. It has been found that the serum of man and many animals frequently contains antibodies, for the production of which there is no evidence of antigenic stimulation. These have been termed *normal or natural* antibodies. It is a well-known fact that transplacental transmission of maternal antibodies to the fœtus takes place; these, however, tend to disappear after 6–12 months of extra-uterine life, and they appear to be different from the natural antibodies. The diversity of the latter does not indicate that such an origin is sufficient to account for all. Their precise origin is, however, unknown, but they probably arise in several ways. In some cases the so-called natural antibodies have doubtless resulted from subclinical infection with various bacteria, in which case the antibodies have arisen as a result of external stimulation of the antibody forming mechanism. Certain antibodies may, however, be produced without any external stimulation; this is evident in the case of the hæmagglutinins, which are responsible for the agglutination of human corpuscles by the serum of other individuals.

How far genetic factors are involved in the production of bacterial antibodies is not known. Some workers have suggested that these antibodies are formed as an ordinary genetic procedure, while others consider that an important factor is that in some individuals the antibody-producing tissues are extremely sensitive to outside stimulation. The relationship of the natural antibodies in providing resistance to infection is uncertain, but it is quite probable that they do participate in the normal defence mechanism.

Antigen-Antibody Reactions. It has been mentioned that the various antibodies are designated by the character of the reaction resulting when union with the corresponding antigen occurs *in vitro*. Several types of reaction occur and they are employed extensively in medicine for diagnostic purposes. Two methods of examination are possible ; in some cases serological tests are used for the identification of an organism isolated from the tissues, *e.g.*, in the examination of members of the typhoid-dysentery group obtained from the fæces, the bacteria to be identified being tested with sera prepared against known antigens. In other instances serum collected from the patient is tested for the presence of antibodies against standardized bacterial suspensions. By such means it is frequently possible to determine the nature of the infection, even though the causative agent has not itself been isolated. The importance of these reactions is thus obvious, and it is consequently necessary not only to be acquainted with the various tests, but also to understand the factors governing them, in order that their application in medicine should be satisfactory. There are several manifestations of antibody-antigen reaction, each of which, together with the reacting substances, has been given a descriptive title. The terms employed are given in Table I.

TABLE I

Antigen-Antibody Reactions

Reaction	Antigenic Material	Antigen	Antibody
Agglutination or clumping.	Bacteria	Agglutinogen	Agglutinin
Precipitation or flocculation.	" Soluble " protein or carbohydrate	Precipitinogen	Precipitin
Lysis or disintegration.	Bacteria	—	Bacteriolysin
Destruction	Bacteria	—	Bactericidin
Complement fixation.	Bacteria, protein	—	Complement - fixing antibodies.
Phagocytosis or intracellular ingestion.	Bacteria	—	Opsonin, Bacteriotropin
Neutralization	Toxin	Toxin	Antitoxin

Agglutination

Agglutination was first described in detail by Grüber and Durham in 1896, when they observed that on mixing a bacterial suspension with the homologous antiserum the bacteria became clumped together. This phenomenon was applied shortly afterwards by Widal to the diagnosis of enteric fever ; on testing the serum of a patient suffering from enteric fever against a suspension of typhoid bacilli agglutination occurred. The agglutination test subsequently became established as a valuable diagnostic measure and it has been applied with success in many other diseases, such as cholera, dysentery, food-poisoning and undulant fever. The agglutination test in enteric fever was for many years frequently referred to as the Widal reaction, in honour of one of the original describers. This practice is now becoming less frequent, as the examination of the serum in such cases is no longer a simple procedure.

In order to demonstrate agglutination, three factors are essential : (1) a suitable antigen, the *agglutinogen*, (2) an antiserum containing the corresponding *agglutinin*, and (3) a satisfactory menstruum. When these factors are present clumping of the bacteria takes place and the resulting clumps are termed the *agglutinate*, which is composed largely of the agglutinated bacteria or cells. The substances, which stimulate the production of agglutinins *in vivo*, are considered to be the same as those which are agglutinated *in vitro* and are frequently termed *agglutinogens*. This term is usually used in a loose sense, as a bacterial suspension almost invariably contains a number of different antigenic factors.

Agglutinogen. It has been noted previously that the bacterial cell does not constitute a simple antigen but has a complex antigenic structure, and it follows that several agglutinogens may be present in a single bacterial cell. This complexity of antigenic structure has only been fully investigated during the past two decades, and it is now recognized that the interpretation of an agglutination test may be a question of some considerable difficulty.

The exact distribution of the various antigenic components in the bacterial cell is unknown, but it is a question of some importance, as those factors situated at or near the surface are more concerned with a reaction than those situated deep in the body-cell. In the case of smooth flagellated organisms which are represented by the symbol " S ", the " H " or flagellar factor is naturally superficial and the "O" or somatic antigens are situated at or near the surface of the cell, while the "Ø" or rough antigen is placed deeper in the cell and is only able to participate in the reaction when the more superficial "O" component is lost. The issue is further complicated in that the change from "S" to "R" is probably a gradual process with various intermediate stages. The suggested arrangement of the main bacterial antigens in a motile organism is represented diagrammatically in the simplest form in Fig. 13.

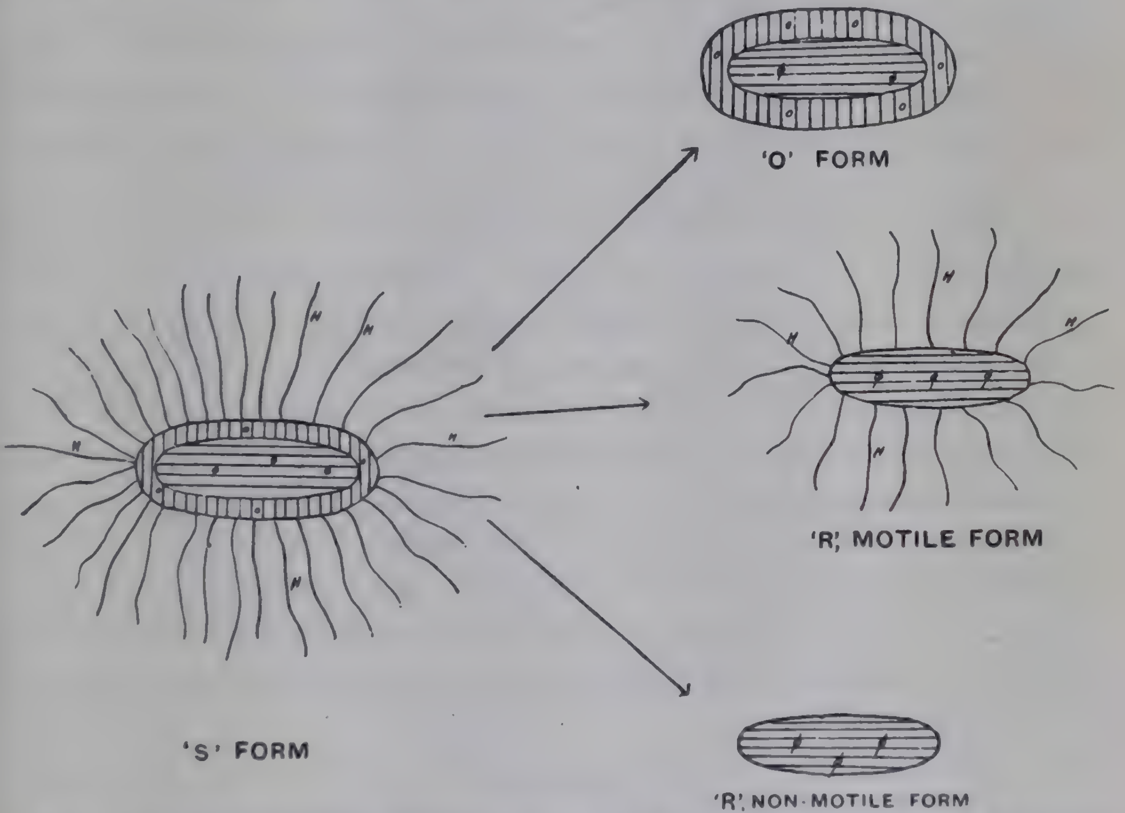


FIG. 13.—Diagrammatic representation of the simple changes in the antigenic structure of a motile organism (*e.g.*, *Bact. typhosum*).

The various bacterial agglutinogens are divided into two main groups according to the nature of the clumps and their time of formation after mixture of the bacterial suspension and serum. Weil and Felix designated these two groups by the symbols "O" and "H". These workers found that flagellated strains of Proteus, giving the floccular type of agglutination, formed a spreading type of growth on the surface of an agar plate, while a few non-motile strains, giving granular agglutination, showed small, discrete colonies. The spreading type of colony was termed *Hauch* = "H", and the compact form *Ohne Hauch* = "O".

The "H" or flagellar agglutinogen is mainly associated with the flagella and is consequently only found in motile bacteria.

It is a relatively labile substance, as it is destroyed by heat over 70°–75° C. and by treatment with absolute alcohol. When mixed with the corresponding agglutinin clumping takes place rapidly in the form of loose floccules.

The " O " or somatic agglutinogen is associated with the body of the bacterial cell. It is more stable than the " H " factor, as it resists a temperature of 100° C. and the action of absolute alcohol. When mixed with the corresponding agglutinin clumping occurs slowly and in the form of small granules, which are not easily broken up by shaking.

Another source of confusion is that the two factors, " O " and " H ", are not simple antigens ; they frequently contain several components which act individually as separate agglutinogens. In view of this multiplicity of antigens, the antigenic structure of a bacterial cell has been likened to a mosaic, but the precise distribution of the various antigenic components has never been determined. Some of the components are often common to several different bacteria and consequently cross-agglutination of different species by one serum is not infrequent. According to the recently introduced classification the different components are represented in the following manner : Roman numerals for the " O " factors, small letters for the specific " H " agglutinogens and Arabic numerals for the group " H " antigens. An example of the overlapping of agglutinogens in different bacteria is seen in the structure of *Bact. typhosum* and a related organism, *Bact. enteritidis* (Table II.).

TABLE II

Antigenic Structure of Related Organisms

Antigenic Components		Bact. typhosum	Bact. enteritidis
" O "		IX XII.	IX XII.
" H "	Specific (phase 1)	d.	gm...
	Group (phase 2).	—	—

Cross-agglutination occurs with these two organisms, due to the presence in each of the common " O " factors, IX and XII ; there is no interaction between the " H " components, neither of which has a group phase.

The antigenic structure is further complicated by the fact that the " H " group of many organisms may exhibit a *diphasic* variation. Andrewes found that, on plating out some bacterial strains and picking off a number of colonies of the same strain, the agglutinability of these was by no means constant. Some behaved as the original culture and exhibited cross-agglutination with allied bacteria, while others only agglutinated in the presence of the specific antiserum. The phases are alternate and the changes reversible. On subsequent investigation this observation was elaborated and the diphasic nature of the bacterial antigens in certain cases, particularly members of the Salmonella group, became established. This diphasic variation was found to involve solely the " H " agglutinogen ; the " O " or somatic agglutonogen remained unchanged. When in the specific phase, allied organisms can be readily distinguished, but in the group phase much antigenic overlapping may take place. A further complication is that some diphasic organisms may possess specific factors in both phases. Thus the Salmonella, *Bact. meleagridis*, has the factors, e and h, in phase 1 and the factors, l and w, in phase 2 ; all these are specific factors and this organism does not therefore possess a true non-specific or group phase.

In considering bacterial variation, the change of bacterial form from smooth to rough was discussed. During this transition the serological reactions undergo a distinct modification : the " O " or somatic agglutinogen is replaced by the " Ø " or rough agglutinogen ; these two antigens are quite distinct and show no specific cross-agglutination. A considerable amount of cross-agglutination is, however, exhibited by the " Ø " antigens of allied strains, *i.e.*, the change from " S " → " R " is accompanied by a definite loss of serological specificity. In motile organisms the " H " factor is unaltered in character, but it tends to decrease quantitatively during this change and is often lost.

Agglutinins. The antibody reacting with an agglutinogen is termed an *agglutinin*. Its exact chemical nature is unknown, but it appears to be closely associated with the globulin fraction of the serum. Each agglutinogen on injection into the animal tissues evokes a separate and distinct agglutinin, which may also be conveniently divided into two main classes, " H " and " O " :—

The " H " agglutinins react with the " H " agglutinogens, but, in contrast to the antigens, they are relatively heat stable and resist heating at 65°–70° C.

The " O " agglutinins react with the " O " agglutinogens and are relatively heat labile, as they are inactivated at temperatures over 65° C.

Menstruum. The nature of the diluting and suspending fluids is of great importance, as the presence of an electrolyte is essential for the reaction. Agglutination is not exhibited when an antigen and the homologous serum are mixed in a salt-free medium. On the other hand, agglutination of bacteria may be accomplished by electrolytes alone, as exemplified by acid agglutination. The concentration and nature of the electrolytes present must therefore be controlled, and in practice it is found that normal or physiological saline provides a very satisfactory medium in most cases. The study of agglutination by rough strains is, however, complicated by the fact that they are usually unstable in physiological saline, being spontaneously agglutinated.

The Mechanism of the Reaction. The phenomenon of agglutination appears to be accomplished in two distinct phases. The first stage consists in the rapid adsorption or fixation of the agglutinins by the agglutinogens. This is usually accompanied, in the case of motile organisms, by loss of motility. The bacteria, modified by the absorption of the agglutinins, are more readily influenced by the physical action of the electrolytes and become clumped; this constitutes the second stage of the reaction. While the action between the agglutinins and the bacteria takes place rapidly, the duration of the second stage is greatly influenced by physical factors. Of these temperature exercises the greatest effect; the velocity of the reaction increases with the temperature up to a maximum of 55° C. This temperature is frequently employed in performing the test. At higher temperatures, 60° C. and over, the agglutinins may be definitely damaged. The presence of living bacteria in the reaction is not essential; bacteria, killed by heat at 56° C., react in a similar manner to the living forms.

Application of the Agglutination Test. The value of the agglutination test as a diagnostic measure in clinical medicine is twofold :—

(A) To determine the nature of an infective process by the examination of the patient's serum against suitable bacterial suspensions.

(B) To establish the identity of a bacterium isolated from the patient's tissues by testing with known antisera.

The test may be carried out either by the slide or tube technique.

The "slide" method is much the inferior test and should be used only for preliminary observations. It is performed by mixing thoroughly a thick bacterial suspension and the antiserum on a slide and subsequent observation through the hand lens or low-power of the microscope. The method is unreliable both as a qualitative and quantitative test and is not to be recommended for general use.

The tube technique is now almost universally used. In this method the bacterial suspension, standardized to the required opacity, is maintained constant and is tested against serial saline dilutions of the serum (1/10, 1/20, 1/40 to 1/10, 240). The test is prepared in small tubes which usually are placed in a suitable rack and incubated in a water bath at 50°–55° C. A temperature of 37° C. may be used, but agglutination occurs more slowly (*vide supra*). Readings are taken after various intervals of time, 1–24 hours, and the result is given as the highest dilution at which clumping of the bacteria is visible. This reading is the titre of the serum. It is usual for constant amounts, 0·25–0·5 c.c., of serum and suspension to be used, therefore the final titre is half the original serum dilution. The readings are most satisfactorily made by observation through a hand lens in a special reading-box. (The detailed technique is given in Chapter XXXVIII.)

It has already been pointed out that the bacterial cells not infrequently contain a multiplicity of agglutinogens and also that different bacteria may contain a common antigenic component. It thus follows that in certain cases a simple agglutination test will not necessarily yield conclusive evidence of the presence of a particular infection or of the identity of the invading bacterium. This difficulty is particularly evident in dealing with the coli-typhoid group of organisms, which possess a multiplicity of antigens and cross-agglutination is very common. When this occurs the identity of the organism is finally established either by the use of sera prepared against the organism when in the specific phase or by means of absorption tests.

Agglutinin-Absorption Test. This test has been employed extensively in the antigenic analysis of the different bacteria. The principle is that common agglutinins may be absorbed by treatment with excess of a bacterial suspension containing the common antigen. The serum, after such treatment, contains the residual antibodies and is more specific; related strains can consequently be differentiated by this means. As an example, the action of antityphoid serum can be examined. This agglutinates suspensions of both *Bact. typhosum* and *Bact. enteritidis*, *Bact. enteritidis* and leaving at 37° C. for seve of the common "O" agglutinins takes pl supernatant fluid after centrifugalization aga suspensions, agglutination now only occurs *typhosum.* The common "O" agglutinins h and removed by the preliminary incubation the serum now only combines with aggluti to the remaining agglutinins *i.e.*, the specific

In special cases when there is difficult organism, *mirror* tests are carried out to te the known and the unknown, is capable of ren from the serum prepared against the other the organisms are obviously identical.

Prozone Phenomenon. It is not infrequ while agglutination is present to a high dil not occur in the first two or three tubes, *i.e.*, greatest concentration of serum. This is t *prozone phenomenon.* The explanation app one, due to excess of one of the reagents, is often seen during the interaction of two Zoning is also encountered in other serologi

Hæmagglutination. Agglutinins are not i inoculation of bacteria. They are also inv of substances, such as red blood corpuscl The agglutination of red blood corpuscles *hæmagglutination* and frequently constitute great importance in medico-legal work.

Agglutinins, capable of agglutinating hu puscles, are normally present in human se uncertain, but there is no doubt that they outside or external stimulation; they follo of heredity. Two main antigenic compon sponding agglutinins are concerned with the to the distribution of these in the blood, groups have been distinguished. Two fa

gens and Group A is consequently sub-
$_1$ and A_2.

TABLE III

Blood Groups

al re.	Antigen (cells)	Agglutinin (sera)
	AB	o
	A	b
	B	a
	O	ab

of great importance in blood transfusion,
possibility of the serious reactions which
on of cells agglutinated by the serum of
ube or tile technique may be used for this
s blood, 3–5 per cent. suspension, is mixed
and anti-B sera, which contain respectively

group O individual agglutinates the cor-
other groups, but not those from the same
hand the corpuscles of a group O person do
lutinogen and are therefore not agglutinated
e persons have consequently been termed
as their blood can generally be transfused
without any harmful effects. The plasma
od is too diluted to exercise any appreciable
the recipient. In practice, however, blood
oup should be used whenever possible and
able to perform a direct test between the
he corpuscles of the donor. The blood of the
e for transfusion when agglutination is not
rtant to note that incompatibility between
e group is occasionally encountered.

laws, any factor found in the child must be present in the parents. The blood of the three parties concerned is examined and the presence of an agglutinogen in the child, but not in either the mother or the suspected father, exonerates the latter from responsibility. A positive result is, however, of limited value.

Two other factors, termed " M " and " N " agglutinogens, were described by Landsteiner and his colleagues in 1928. These substances are quite distinct from the " A " and " B " factors. Agglutinins to the " M " and " N " agglutinogens do not occur naturally and these factors consequently do not require consideration in the selection of donors for blood transfusion.

The " M " and " N " factors define three separate blood groups, viz., " M.M.", " N.N." and " M.N.", one or both factors being always present. The " M " and " N " agglutinogens are demonstrated by the use of sera prepared in animals, usually rabbits. The preparation of specific sera is difficult, as all traces of " α " and " β " agglutinins must be removed by absorption before satisfactory results can be obtained. This is even necessary when blood of group O, which contains neither the " A " nor the " B " antigenic factor, is used for immunization.

The " M " and " N " agglutinogens, as with the " A " and " B " factors, are inherited according to strict Mendelian laws and therefore extend the value of blood tests in disputed paternity cases ; twelve main blood groups can now be defined. In carrying out the tests fresh sera and satisfactory controls must be available as these sera tend to deteriorate rapidly in the laboratory.

A further agglutinogen has recently been identified ; this is known as the Rh or Rhesus factor because the agglutinogen was first demonstrated in the erythrocytes of the rhesus monkey. The Rh factor is found in some 85 per cent. of human beings ; the corresponding agglutinin is not normally found. Rh negative individuals can, however, form *rh* or anti Rh agglutinins if the agglutinogen is introduced into their circulation, as following transfusion with Rh positive blood, or, in the case of a woman, if she becomes pregnant with an Rh positive fœtus. Serious reactions may occur under such circumstances. It has been found that, in some 90 per cent. of infants affected with erythroblastosis fœtalis, the mother is Rh negative and the infant Rh positive. Sub-types of the Rh agglutinogen have been described but their detection is a highly specialized procedure.

Agglutinins and Immunity. Agglutinins are formed either as a result of the injection of vaccines or following a definite infection with a bacterium. The response varies both with the nature of the antigen, " H " agglutinins being more readily evoked than " O " agglutinins, and also with the species of animal concerned, for example, rabbits respond more readily than guinea-pigs. As agglutinins can be detected without much trouble, the following question arises: do agglutinins provide an index of immunity? This problem has received much attention; experimental animals have been given prophylactic injections of vaccines and the appearance of the various agglutinins has been compared with the resistance offered to subsequent infection with the virulent homologous organism. By such means it has been established beyond question that the two classes of agglutinins differ considerably. The " H " agglutinins are valueless as an index of immunity; animals whose sera give high titres of " H " agglutinins usually exhibit no more resistance to infection than uninoculated animals. This finding was doubtless responsible for the view of earlier workers that agglutinins were of no value as an index of immunity, as all the early agglutination tests with motile organisms were concerned almost entirely with the " H " factor. Much evidence has, however, accumulated to indicate that the presence of " O " agglutinins in the serum is associated with increased resistance against the homologous organism. It has been shown repeatedly that animals injected with " O " antigens are definitely more resistant to subsequent infection than either uninoculated animals or those receiving mainly " H " antigens. The variation from smooth to rough, during which the " O " antigen is changed, is also accompanied by a loss of immunogenic power of the bacterium.

The function of agglutination as a protective mechanism is not known. The bacteria are not killed, but it would appear that, when clumped, they tend to remain localized and are thereby more readily attacked by the defensive forces.

Felix and Pitt have recently described another antigenic component of *Bact. typhosum*, which is extremely difficult to identify. Certain inagglutinable strains of *Bact. typhosum* were found to be particularly virulent for mice, and it was considered that their virulence was intimately associated with the resistance to the action *in vitro* of the " O " antibody. The peculiar behaviour of these strains was shown by agglutination and absorption tests to be due to the presence, apparently on the surface, in virulent strains of *Bact. typhosum* of a separate antigen, to which they gave the symbol " Vi ". The nature and biological distribution of the new antigenic factor are obviously matters of considerable importance, but as yet it has been demonstrated only in virulent strains of certain Salmonella organisms. It is a relatively labile substance, being destroyed by boiling and phenol, but it develops when a virulent strain is grown on soft ascitic agar over a comparatively narrow temperature range at about 37° C. These strains are practically inagglutinable by an " O " antiserum and resist phagocytosis. The " Vi " antigen appears to be of great importance in both active and passive immunization. Experimental evidence indicates that the " Vi " antibody has a powerful protective value, and trials are being made to determine its therapeutic value in enteric fever; the results so far obtained are considered to be promising. It is, however, incapable of neutralizing the endotoxin produced by the organism, this being the function of the " O " antibody. The serum of enteric fever patients and convalescents often contains the " Vi " antibody, which, it is claimed, is also present in the serum of carriers of *Bact. typhosum.* Developments in this field will be awaited with interest.

Precipitation

The phenomenon of precipitation was first described in 1897 by Kraus, who observed that when antisera, prepared against the typhoid bacillus, the cholera vibrio and other bacteria, were mixed with bacteria-free filtrates of broth cultures of the homologous organism a macroscopic precipitate was formed. These observations were soon confirmed, and it was subsequently found that the reaction was produced not only by bacterial proteins, but also by all proteins and carbohydrates having a sufficiently complex molecular structure. The reaction is termed *precipitation*, the antigen the *precipitinogen* and the antibody the *precipitin.*

Precipitation is considered to be analogous to agglutination. The difference between the two reactions is in the nature of the antigen; in agglutination this is a suspension of relatively large particles, while in precipitation it is in an extremely fine colloidal solution. In agglutination the agglutinate is formed largely from the antigen suspension, whereas in precipitation the precipitate is formed mainly by the serum-globulins, *i.e.*, the antibody. The precipitate once formed is not always insoluble, it may readily pass into solution by some alteration in the physical conditions of the test. It has been found that a temperature of 55° C. may cause this to happen, and incubation at this temperature is consequently not usually employed for the test.

The technique used for the quantitative test is necessarily different from that required for demonstrating agglutination. The precipitate is largely formed by the precipitin and serial dilution of the antiserum is therefore unsatisfactory, as the power to form a precipitate becomes lost on increasing the dilution. In order to overcome this difficulty, the antigen is subjected to serial dilution and is tested against a constant amount of the antiserum. The mixtures are either incubated at 37° C. for several hours or left at room temperature or 0° C. overnight when readings are made. Zones of inhibition are frequently encountered in this reaction. Dean and his co-workers have found that there is a definite ratio between the amounts of antigen and antibody present in the reaction at which precipitation is most marked and occurs most rapidly. The application of this method of determining the optimal proportions of the reagents in the test has proved of great practical value. It is used for estimating the amount of precipitin in a given serum and also in the titration of the different antitoxins.

Precipitation, owing to its great delicacy and specificity, is used in medico-legal work to determine the origin of blood stains and other animal proteins. Antisera prepared against the serum of different animals are tested against saline extracts of the material under examination ; the appearance of a precipitate with one of the sera indicates its origin. The various types of the pneumococcus can also be identified by means of the precipitation test ; the specific soluble substance of the pneumococcus is a *haptene*, *i.e.*, it is able to react *in vitro* with the homologous antiserum. Precipitation can sometimes be obtained by testing the urine of cases of pneumonia with the homologous type antiserum.

Bacteriolysins and Bacteriocidins

The bactericidal action of blood was first noted by Nuttall in 1888, when he found that, after contact with defibrinated blood, the growth of anthrax bacilli became less marked. It was later shown that this action was exercised by serum alone, but was prevented when the serum was heated at 56° C. for 30 minutes. The phenomenon was, however, first fully described by Pfeiffer during his research on the cholera vibrio. Pfeiffer followed the course of events when cholera vibrios were injected intraperitoneally into immune guinea-pigs and found that the vibrios first lost their motility, then became swollen and granular, and finally underwent disintegration. A similar sequence followed the intraperitoneal inoculation of normal pigs with vibrios mixed *in vitro* with immune serum, even when this had been heated at 70° C. for 1 hour. This extracellular lysis of the cholera vibrios was termed specifically *Pfeiffer's phenomenon* and was the first demonstration of the reaction, *bacteriolysis*. Metchnikoff and Bordet subsequently found that bacteriolysis occurred *in vitro* when either fresh peritoneal exudate or normal serum was added to heated immune serum; the heated immune serum alone had no apparent action. This reaction was, however, only obtained with a limited number of organisms, particularly certain of the Gram-negative bacilli.

These and numerous other observations indicated that two factors are involved in the reaction. One is relatively heat stable, withstanding heat at 56°–70° C. for ½ hour; it was termed *amboceptor* by Ehrlich and the German School, and *substance sensibilisatrice* by Bordet and the French School; it is now usually referred to as a *lysin* or *immune body*. The second factor is relatively heat labile, being destroyed by heat at 56° C. in 10–20 minutes, and is present in normal serum. This substance was designated *alexine* by the French School and *complement* by Ehrlich; the latter term, *complement*, is the one now generally employed.

In other instances, as a result of a similar reaction, the organisms were killed without undergoing lysis. The antibody responsible for this bactericidal action has been termed a *bactericidin*.

Bordet found that the phenomenon of lysis was not restricted to bacteria, but was also shown by various tissue-cells. Red blood corpuscles in the presence of an appropriate antiserum were readily lysed; this reaction was an example of *cytolysis*, and was termed more specifically *hæmolysis*. Hæmolysis is very simple to demonstrate and, as bacteriolysis and hæmolysis are closely related, we owe much of our knowledge of the subject to observations made on the latter phenomenon. Numerous investigations have been carried out in attempts to discover the mechanism of the reaction and much information is now available. The reaction is more complicated than agglutination and precipitation as three, in place of two, variable factors are involved, and it is not surprising that many theories as to the mode of action have been suggested. Ehrlich, in designating the immune body by the term *amboceptor*, applied his side-chain theories to the reaction. He considered that the immune body had receptors for both antigen and complement, and through its intervention complement became combined with the antigen. Ehrlich's views had the important effect of stimulating work on the subject, but apart from this they are now of doubtful importance. It has been found that complement cannot act on an antigen which has not been previously sensitized, nor does it unite with the immune body alone. Facts at present available support most strongly the conception of Bordet and indicate that the immune body acts on the antigen in a physico-chemical manner, probably on the surface, and renders the antigen susceptible to the action of the complement, *i.e.*, the antigen is sensitized by the immune body.

Complement. Complement is a complex substance, the chemical nature of which has not been determined. It has antigenic properties and this suggests that it might be a protein. Experimental evidence indicates that it has four distinct components, two of which are inactivated at 56° C. while the other two are more heat stable. It is present in the normal serum of all animals, but the amount varies with the different species. It appears to be most marked and constant in the guinea-pig, which is the usual source of complement for laboratory purposes. It is non-specific; complement which brings about bacteriolysis appears to be identical with that taking part in hæmolysis. It is not increased by any process of immunization. Complement is thus not a true antibody. Its source is uncertain, but it is probably formed by the body-cells, particularly the leucocytes. Although complement is very labile and deteriorates rapidly at room temperature, it may be conserved in a frozen state for several days and in the dried state for long periods.

Immune Body or Lysin. The immune body is a true antibody. It exhibits a high degree of specificity and is increased by immunization with the homologous antigen. It is relatively heat stable, as it withstands a temperature of 60°–70° C. for ½ hour. It combines with its corresponding antigen at temperatures as low as 0° C., and this combination is to a certain extent reversible. Complement, on the other hand, cannot combine with the sensitized antigen at 0° C., but does so readily at 37° C.; this combination is not reversible.

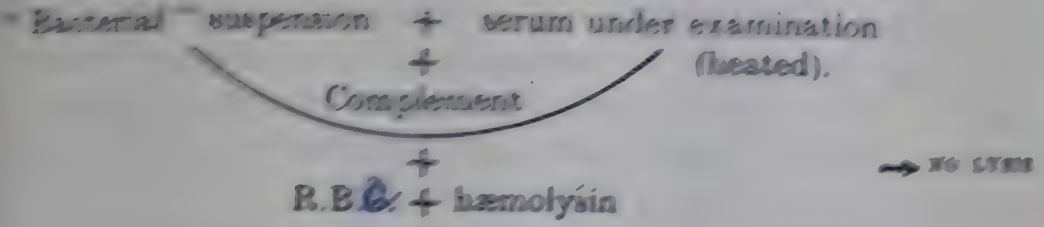
There is a quantitative relationship between the immune body and complement required to complete the reaction. Within limits by increasing the amount of one, there is a decrease in the amount of the other required, *e.g.*, in the phenomenon of hæmolysis it has been found that, up to a certain point, the more the cells are sensitized the smaller the quantity of complement required to produce complete hæmolysis.

Conclusive evidence has not been obtained to show that complement and immune body interact in the absence of antigen. Neisser and Wechsberg (1901) found that the bactericidal action of immune serum was impaired by the presence of excess of immune body. This was termed the *Neisser-Wechsberg phenomenon* and was considered to represent a deviation of the complement by the superfluous immune bodies. This explanation is, however, purely theoretical. In the absence of evidence indicating union of complement and immune body alone, the indications are that the inhibition is merely due to a zoning phenomenon, such as is found in other serological reactions.

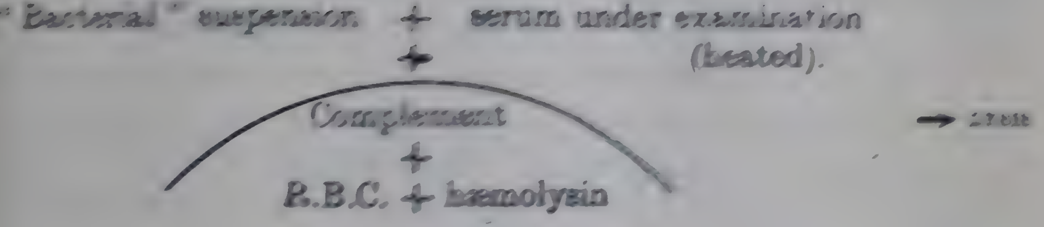
Complement-Fixation Test

In view of the fact that bacteriolysis and hæmolysis were so closely related, Bordet and Gengou (1901) considered that hæmolysis might be employed as an indicator of the absorption of complement by a bacterium and its homologous immune body. They consequently introduced the serological reaction known universally as the complement-fixation test; some workers have erroneously termed it the complement-deviation test. The original technique of this test has been subjected to many modifications, but the essential principles are unaffected. The salient features are as follows: the bacterial suspension is mixed with the serum under examination and complement; this mixture is allowed to react for a given time, at the end of which red cells and the corresponding hæmolysin are added and the whole incubated at 37° C. for a stated time. If the serum under examination contains the homologous immune bodies, complement is taken up during the first incubation period and consequently lysis of the sensitized red cells does not take place. On the other hand, if immune bodies are not present in the serum, complement is free and is therefore available for the lysis of the cells. This reaction is given below in a diagrammatic form:—

(1) Positive reaction.



(2) Negative reaction.



It is seen that five factors are involved in the reaction, and as only one unknown is practicable in diagnosis, it is essential that the remaining four should be standardized previous to the test.

(1) In the preparation of the *bacterial suspension* it is important to note that this frequently absorbs a certain amount of complement non-specifically, *i.e.*, without previous sensitization. It is necessary, therefore, to test the suspension for *anti-complementary activity*, as this phenomenon is termed. A suspension taking up an appreciable amount of complement is unsatisfactory and should be discarded.

(2) The *red blood corpuscles* are collected, usually from sheep, a short time before use, as if kept they tend to exhibit autolysis. The blood, during collection, should be prevented from clotting either by defibrination or the addition of a citrate solution. The cells must be well washed with saline before use. After these preliminary precautions a suitable strength in saline is prepared; the strengths in general usage are 5 per cent. and 10 per cent.

(3) Owing to the rapid deterioration of *complement*, it should be used within 24 hours of collection unless it has been preserved in the frozen or dried state. Guinea-pig serum provides the most convenient source, but before use in the test it is always necessary to titrate the amount of complement present. This is done by determining the highest dilution which, in the presence of excess of hæmolysin, will cause complete lysis of the standard suspension of red cells in 1 hour at 37° C. This dilution is said

to contain 1 minimal hæmolytic dose (M.H.D.) of complement. In practice, 2, 3 or 4 M.H.D. of complement are usually employed.

(4) The *hæmolysin* is prepared by immunizing rabbits with sheep R.B.C. over a period of time; when a suitable titre has been obtained the rabbit is bled, the serum collected and stored in sterile bottles at 0° C. Owing to its marked stability, the hæmolysin keeps indefinitely under such conditions. It is titrated in a similar manner to complement: the M.H.D. of the hæmolysin is the least amount causing complete lysis of the R.B.C., in the presence of excess of complement, in 1 hour at 37° C. With a satisfactory serum this may represent a dilution of 1/15–30,000. In the test 2, 3 or 4 M.H.D. are frequently used. The possibility of complement being added with the hæmolysin is eliminated usually by the high dilution at which this serum is used and also by the prolonged storage to which the serum is often subjected. In order to remove all doubt, the hæmolysin may be heated at 56° C. for ½ hour before use.

(5) The serum under examination is heated at 56° C. for ½ hour to destroy any complement that may be present. This serum may exhibit marked anticomplementary properties and strict controls are necessary.

By adjustment of the various factors a qualitative serological test of marked specificity is available. If a quantitative examination is also required, this is performed by varying the amounts of either the serum, complement or hæmolysin in the test.

Whenever combination of an antigen and its corresponding antibody occurs, complement, if present, is fixed. Complement is thus taken up in many serological reactions; in the precipitation test fixation of complement can even be demonstrated, although there may be no visible precipitate.

Complement fixation may be applied in practice in two ways: (*a*) with a known antigen and unknown serum, and (*b*) with an unknown antigen and known serum. The former application, (*a*), is used extensively in the diagnosis of syphilis under the title of the " Wassermann reaction ".

It has been found that human serum frequently contains a substance capable of sensitizing sheep corpuscles to the action of complement. This substance is termed a *normal or natural hæmolysin*. It does not usually interfere with the application of complement fixation in diagnosis, but in the presence of certain antigens complement may be fixed, in which event a source of irregularities is present. The antigens reacting with these hæmolysins are of a lipoidal nature and are often found in various organs of many animals, including the guinea-pig, horse and dog, but not man, rabbit and sheep. These antigens were originally found by Forssman to stimulate the production of antisheep hæmolysin on inoculation into rabbits, and they have been termed *Forssman* or *heterophile* antigens. The possibility of their participating in the complement-fixation test is avoided by the use of antigenic suspensions, such as sheep or human heart-muscle extracts, which do not contain these heterophilic elements.

Phagocytosis, Opsonins and Bacteriotropins

Following the original observations of Metchnikoff on phagocytosis and his contention that the ingestion of bacteria by the body-cells was the principal factor of the defence mechanism against bacterial invasion, this reaction became the subject of much investigation. Although it had previously been shown that a factor present in the serum was essential for phagocytosis, the mechanism of the reaction was finally and conclusively worked out by Wright and his colleagues in the early years of the present century. Wright found that two factors were necessary for the ingestion of bacteria : (1) cells and (2) serum. The cells involved in the reaction had been grouped by Metchnikoff into two classes : (*a*) the microphages, which consisted mainly of the polymorphonuclear leucocytes, and (*b*) the macrophages, which included the large mononuclear and endothelial cells. Wright, however, demonstrated that, when the leucocytes were separated from the serum and washed well with saline, they were unable to ingest bacteria. On the addition of fresh serum to the cells or by allowing the serum to act first on the bacteria, ingestion of bacteria resulted. It was thus demonstrated that some substance present in normal serum was required for phagocytosis, and this body was found to be inactivated by heating the serum at 55° C. As it apparently prepared the bacteria for ingestion by the cells, Wright termed it *opsonin*.

In certain coccal infections the ability of the leucocytes to ingest the homologous bacteria is subject to much variation. This variation is largely governed by the activity of the opsonins. In an attempt to estimate the resistance to coccal infections, the number of bacteria ingested by cells plus different sera is measured ; from this a figure known as the *opsonic index* is estimated. Using a standard bacterial suspension, leucocytes from a normal individual, normal serum and the serum under examination, the following mixtures using unit volume of each are prepared :—

(1) suspension + normal leucocytes + normal serum ;
(2) „ + „ „ + patient's serum.

These mixtures are incubated at 37° C. for 15 minutes, after which smears are made on separate slides ; these are suitably stained and the number of bacteria contained in 500 leucocytes is counted on each slide. An average per leucocyte is made, and the ratio of the average given by the patient's serum to that given by the normal serum is the *opsonic index*.

The opsonic index was used for some time as a means of evaluating vaccine therapy in coccal infections. Owing to the number of variable factors involved and the inherent inaccuracy of the test, this estimation is now considered to be of little practical value.

Shortly after the observations of Wright were published, Neufeld and Rimpau found that in antipneumococcal and antistreptococcal sera there were substances having a similar action to the opsonins ; they were, however, not inactivated by heat at 60° C. for 30 minutes. These substances were considered to be distinct from opsonins and were termed *bacteriotropins*. These observations were soon confirmed, and it was found that heat-stable opsonic substances were produced by immunization with other bacteria or such antigens as red blood corpuscles.

The relationship between opsonins and bacteriotropins, or immune opsonins, has been the subject of much conjecture. Attempts have been made to identify opsonins with complement, as both are present in normal serum and also both are relatively heat labile. While the results have been irregular, it seems probable that opsonins are quite distinct from complement. It has been shown with normal serum that, although some of the opsonin action is non-specific, there is, nevertheless, a certain degree of specificity of action. The available results indicate that bacteriotropins are opsonins evoked by the immunization process, and also that, in the opsonification of bacteria, complement takes a part. In normal sera, as the opsonin content is relatively low, the complement takes a correspondingly larger part in the reaction. In the case of immune sera bacteriotropins are present in greater concentration, and therefore the part played by complement is of little significance. In this way the greater specificity exhibited by immune sera, as compared with normal sera, is explained.

Bacteriotropins are thus specific immune bodies, which are identical with other thermostable antibodies, such as the bactericidins, agglutinins and precipitins. Opsonins belong to the group of thermolabile natural antibodies.

Phagocytosis appears to be one of the most important defence mechanisms against invasion by bacteria, such as the Gram-positive cocci, that do not undergo extracellular lysis. It is also largely responsible for the removal of inert particles introduced into the tissues. In some cases phagocytosis may be responsible for the spread of infection. Bacteria contained in the cell may be conveyed to other parts of the body and, instead of being destroyed by the cell, they may multiply, kill the cell, become liberated and produce a local lesion. The intracellular position of bacteria does not necessarily indicate that they are dead.

The mode of action of opsonins and tropins is not fully understood. It is probable that they act on the surface of the bacteria and so render them more readily ingested by the phagocytes.

Toxin-Antitoxin Neutralization

The reactions which have just been considered, with the exception of precipitation, involve the bacterial cell. In the case of those bacteria which, under suitable conditions, produce active toxins a further serological reaction is manifested. Toxins on injection into the tissues of laboratory animals stimulate the production of corresponding antibodies, which are termed *antitoxins*. The importance of antitoxins in increasing the resistance offered to infection with the corresponding organism was first demonstrated by Behring and Kitasato in 1890, during their experiments with the tetanus bacillus. It has since been shown that antitoxins may be produced by the inoculation of suitable animals not only with bacterial toxins, but also with the poisonous substances found in plants, such as ricin and abrin, and those produced by venomous snakes. One of the most important properties of antitoxins is their ability to neutralize the action of the homologous toxin. This can be shown in the following manner—appropriate mixtures of toxin and antitoxin, after standing *in vitro* for definite periods of time, are injected in a suitable manner into a satisfactory experimental animal, such as the guinea-pig or mouse. In order to provide a control, a similar mixture is prepared in which saline is substituted for the serum and the same procedure adopted. The control animal should exhibit characteristic signs of toxæmia, whereas, if the serum contains sufficient antitoxin, the test animal should remain unaffected. This test is termed a *neutralization test,* and is the one largely employed at the present moment to determine the potency of either a toxin or an antitoxin. In each case the other component of the test must be standardized previously. The route of inoculation of the mixture varies, but as a general rule the mixtures are injected either intradermally or subcutaneously.

The Neutralization Test. As already noted, the essential feature of the test is the neutralization of toxin by antitoxin. The mechanism of the reaction has been subjected to much investigation and many theories of the mode of action have been propounded. While the phenomenon is not completely understood and controversial points arise, there are certain facts which appear to be quite definite. Thus it has been determined that the toxin is not destroyed by antitoxin *in vitro ;* the action is reversible, combination occurs but this may be broken down by various physical and chemical processes, such as dilution, heating, freezing and treatment with weak acids. The union becomes more stable by increasing the time of contact *in vitro,* but nevertheless, no matter how long the duration of the contact, dissociation can always be accomplished to some extent. Failure to appreciate the possibility of dissociation in toxin-antitoxin mixtures has had serious consequences. Mixtures of diphtheria toxin and antitoxin, originally neutral and containing a small percentage of phenol as a preservative, were stored for some time at low temperatures and became frozen ; on subsequent injection into children several deaths resulted. It appears that as a result of freezing the phenol had become concentrated locally and had destroyed the antitoxin without damaging the toxin.

Danysz established the important fact that the amount of toxin neutralized by a fixed amount of antitoxin varied according to the manner in which the toxin was added. When it was added in bulk more was neutralized than when the toxin was added in separate fractions. This reaction has been termed the *Danysz phenomenon.*

The three most important theories of toxin-antitoxin neutralization are those propounded by Ehrlich, Arrhenius and

Madsen, and finally Bordet. Ehrlich evolved his side-chain theory mainly from observations on the neutralization of diphtheria toxin by the antitoxin. Although his views cannot be accepted in the light of present knowledge, they were nevertheless responsible for stimulating research on the problem. Various units were determined during Ehrlich's work :—

(1) M.L.D. (minimal lethal dose) = the smallest amount of toxin killing a 250-gm. guinea-pig in 4 days after subcutaneous injection.

(2) Antitoxin unit = smallest amount of antitoxin neutralizing 100 M.L.D. of toxin.

(3) L_0 (*Limes nul*) dose = largest amount of toxin which is just neutralized by 1 unit of antitoxin.

(4) L_+ (*Limes tod*) dose = smallest amount of toxin killing a 250-gm. guinea-pig in 4 days when mixed and injected with 1 unit of antitoxin.

Ehrlich considered that toxin-antitoxin neutralization was analogous to the reaction between strong acids and alkalies. In order to explain certain irregularities, Ehrlich postulated that the toxin consisted of two elements, a toxic or toxophore receptor and a binding or haptophore receptor. In the case of toxins which had lost their toxicity but retained their capacity to bind antitoxin, he stated that the toxophore receptor had degenerated, and termed this modified toxin *toxoid*. It was reasonably expected that, with any given toxin, the value, L_+ dose $-$ L_0 dose, would be equal to 1 M.L.D. On examination, however, this value was found to be considerably greater, in some cases it was as much as 50–100 M.L.D. This was termed *Ehrlich's phenomenon*. The explanation offered by Ehrlich to explain this discrepancy was that in any crude toxin toxoids were always present, and, when the L_0 dose of toxin was mixed with 1 unit of antitoxin, both toxin and toxoid combined with the antitoxin. On the addition of more toxin, this displaced a corresponding amount of toxoid from combination with antitoxin, as the toxin had a greater affinity than toxoid for antitoxin. Thus in order to obtain the L_+ dose of toxin, all the combining toxoids had to be replaced by toxin ; a much greater amount of toxin than the L_0 dose $+$ 1 M.L.D. was consequently required.

In spite of the ingenuity of Ehrlich in devising new theories to explain this and other phenomena, his views were subjected to much criticism. Arrhenius and Madsen put forward the view that the toxin-antitoxin reaction was closely allied to the reaction between weak acids and alkalies. They compared their results with those obtained during the reaction between boric acid and ammonia, in which an equilibrium is established according to the law of mass action. This chemical theory does not, however, explain satisfactorily the Danysz phenomenon or the limited degree of dissociation of toxin-antitoxin mixtures following dilution.

Bordet suggested a more probable theory, which was mainly founded on physical grounds; it explained the various phenomena on the basis of adsorption. A chemical factor was later introduced to explain specificity. The smaller toxin molecule is considered to be adsorbed on the surface of the larger antitoxin molecule. *Antitoxin-toxin* neutralization was thus analogous to the unions of two colloids, and he compared it with the adsorptive action of filter paper on dyes. If the intact filter paper is added to the dye, it becomes stained uniformly; if, however, the paper is torn in strips and is added strip by strip, the first strips will be stained much deeper than the last ones.

While the adsorption theory of Bordet appears the most satisfactory and offers a reasonable explanation for Danysz's and Ehrlich's phenomena, it does nothing to explain the marked specificity of the reaction. It may probably be that some other factor is also involved in the reaction, but definite evidence of this is lacking.

The Standardization of the Reagents. The great importance of antitoxin in passive immunization and the recent advance in active prophylaxis with toxins and toxoids indicate the necessity for the accurate standardization of these substances. This is a matter of some difficulty, owing to the variable factors involved, and it is doubtful whether reliance should be placed on any single method.

Ehrlich, as previously noted, introduced a number of arbitrary units, some of which are still employed for titration purposes.

The M.L.D. of a toxin was the unit used extensively by Ehrlich, but it is now considered a very unsatisfactory standard. It is known that toxins lose their toxicity, but retain their combining power on keeping; the use of this unit as a basis of standardization is consequently liable to produce irregularities. Antitoxic serum shows little variation in activity over periods of time, particularly if preserved in a dried state, and it has been found to constitute a more suitable reagent as a basis for standardization.

The unit which is now employed extensively for standardization is consequently the L_+ or *Limes tod* unit of Ehrlich. This is defined as the smallest amount of toxin which, when mixed with

1 unit of antitoxin, will, on subcutaneous inoculation, cause the death of a 250-gm. guinea-pig within 4 days.

In testing a new batch of antitoxin, standard samples of antitoxin are obtained from the official Standards Laboratory. The standard antitoxin is conserved in a dried state and was originally standardized directly or indirectly against Ehrlich's standard serum. The L_+ dose of a ripened toxin is obtained by using the standard antitoxin; then using this dose of toxin, the amount of the fresh antitoxin necessary to give the same result is estimated. This amount will contain 1 antitoxin unit; the number of units in 1 ml. of the serum can then be readily estimated. By the adoption of this method of standardization the results obtained in different laboratories are within certain limits comparable. In some cases the intradermal method of standardization is employed, as it has the important advantage that several observations can be made on one guinea-pig. The intradermal injection of a small dose of diphtheria toxin gives rise to a marked local lesion; the addition of antitoxin to the toxin prevents this. Two further units or doses are employed in this method. The M.R.D., or *minimal reacting dose*, of a toxin is the smallest amount, when injected intradermally into a guinea-pig, giving the characteristic local reaction. The $\mathbf{L}_r$ dose of toxin is the smallest amount of toxin which, when mixed with 1 unit of antitoxin, will give rise to the typical local lesion on intradermal injection into a guinea-pig.

Titrations may also be carried out by the flocculation test of Ramon, which is a specific precipitation in which toxin and antitoxin participate. The appearance of rapid maximal flocculation is observed in the presence of 1 unit of antitoxin; this gives us another unit, the $\mathbf{L}_f$ dose of toxin. This method is used extensively in France, but it has a more restricted application in this country where it is used as a preliminary measure to animal tests. Flocculation is less reliable than the other methods, as antitoxin gives a reaction with both toxin and toxoid, and it is mainly a test of combining power.

Antitoxins and Immunity. The presence of antitoxins in the serum of an individual is an indication of increased resistance to infection with the homologous organism. If the bacterium is dependent for its pathogenicity mainly on its ability to produce a toxin, the neutralization of the toxin before it has reached the susceptible cells is sufficient to keep the infection in check. An example of this is diphtheria, in which condition the presence of antitoxins in the serum tends to prevent or limit the infection.

When, however, the toxin is only part of the offensive mechanism of a bacterium the action of antitoxins is less marked. The toxic activity is checked, but the bacterial activity is only slightly restrained. This is illustrated in the case of some streptococcal infections, in which the administration of antitoxic sera has little effect on the local pyogenic lesions. It also offers a probable explanation of the apparent ineffectiveness of antitoxic sera in infections with *gravis* strains of diphtheria bacilli.

Unity or Diversity of Antibodies

The relationship between the different antibodies has been widely discussed. Many serological tests have been described and, according to the type of reaction involved, the reacting antibody has received a descriptive title, *e.g.*, agglutinin, precipitin, bactericidin, bacteriolysin, bacteriotropin, opsonin and antitoxin. From a superficial survey of the question it is, however, difficult to conceive that one single antigen should stimulate the production of such a large number of separate distinct antibodies. In the early days of bacteriology and immunology the view was held that all antibodies were distinct entities. This doctrine was sponsored and upheld by Ehrlich, who devised new side-chain theories to explain any fresh serological reaction. This hypothesis became more and more discredited as the physical theory of adsorption advocated by Bordet gained ground. The analogy between colloidal reactions and antigen-antibody reactions suggested that the various names given to the antibodies did not necessarily indicate that a distinct body was present in the serum.

It has already been noted that, in the various reactions, two stages occur : the first comprises the union between the antibody and antigen, the second is the actual reaction which is governed largely by the physico-chemical structure of the antigen and the conditions under which the reaction is taking place. This does not suggest that the nature of the antibody determines the reaction, but that this is a secondary phenomenon governed by non-specific factors. This view of unity of antibodies was investigated at length by Dean and his colleagues, who demonstrated that precipitation and complement fixation were dependent on a single antibody, and that fixation of complement occurred during the early stages of precipitation.

Zinsser also obtained evidence to support the unitarian view, but emphasized that this does not imply that a bacterium contains a single antigen. It merely indicates that a single antigen produces a single antibody. Union between the two components in a serological reaction occurs rapidly ; if electrolytes are present and the antigen is soluble, flocculation occurs, while an antigen of cellular structure under the same conditions gives rise to agglutination. Some bacteria, particularly the Gram-negative bacilli, become sensitized to the action of complement and undergo extracellular lysis. Other organisms, such as the Gram-positive cocci, do not undergo extracellular lysis ; after sensitization they become more readily ingested by the phagocytes and may undergo intracellular lysis.

There is thus much evidence to support the unity of antibodies. In the present state of our knowledge the antibodies may be conveniently divided into two classes, one containing the antitoxins and the other the remaining antibodies, which, although they have received different names, are dependent on a single form of antibody. This does not necessarily mean that these two groups are quite separate and distinct. The flocculation resulting from union of toxin and antitoxin in the Ramon test indicates that the two classes may be related.

CHAPTER XI

HYPERSENSITIVENESS: ANAPHYLAXIS, IDIOSYNCRASY AND ALLERGY

HYPERSENSITIVENESS indicates a susceptibility to substances which give rise to little or no reaction in normal members of the same species. It is a phenomenon closely related in some of its aspects to immunity, as the indications are that the various reactions result from a union *in vivo* of an antigen and its corresponding antibody. It differs, however, from the ordinary immunological reactions in that the reaction can only be demonstrated in the living tissues and, also, in that the resistance of susceptible individuals is not apparently increased by the injection of the foreign substance, but the tissues are rendered highly sensitive to subsequent inoculations of the same substance.

The increased reactivity of the tissues may follow the introduction into them of a large variety of substances and may be manifested in many diverse forms. The signs of hypersensitiveness are largely dependent on the host species, the nature of the responsible substance and the route of its administration. In view of the marked variability of the reaction, it is not surprising that there has been much confusion in the nomenclature and classification of the condition. Independent workers have described individual phenomena and, in some cases, have introduced special terms to designate them. In recent years it has become more and more recognized that the various manifestations are closely related. It is, however, convenient to arrange the reactions in sub-groups, and the following arbitrary scheme, while only tentative, is probably the most suitable of those suggested :—

1. *anaphylaxis*, which is hypersensitiveness produced experimentally in animals,
2. *idiosyncrasy*, which includes the various human forms of hypersensitiveness, such as serum-sickness, asthma, hay fever, urticaria, etc.,
3. *allergy*, which is the type developing during a bacterial infection.

There is, however, no absolute differentiation between these various groups ; moreover, the mechanism of these reactions involves an antigen-antibody union *in vivo* and is closely related to that of immunity, any differences that may exist are quantitative rather than qualitative. In all cases there is a reaction of a defensive nature to the introduction of a foreign substance into the tissues ; in hypersensitiveness, however, unpleasant symptoms are frequently produced by the reaction. In the case of anaphylaxis certain differences, particularly the severity of the symptoms, may be due to the fact that it is purely an experimental condition and does not occur spontaneously.

Anaphylaxis

Attention was first directed to the phenomenon of hypersensitiveness by the observations of Richet and his colleagues in the early part of the present century. They found that, during the repeated injection of an extract of the tentacles of actiniæ into dogs, if a certain time interval were allowed between the first and second doses definite symptoms, followed sometimes by death, appeared. This increased sensitivity, which was found after the initial dose in place of the expected protection, suggested the term *anaphylaxis* (= against protection) for this phenomenon to indicate a contradistinction to *prophylaxis*.

Soon after this observation Arthus (1903) described a curious local reaction following repeated subcutaneous inoculations of horse serum into the rabbit. While the early injections produced little or no effect, subsequent injections gave rise to a marked local reaction. This became more intense with each further injection and consisted of an œdematous mass with considerable local necrosis. This reaction has been termed the *Arthus phenomenon* and is an example of a local manifestation of a general hypersensitivity. Phenomena associated with hypersensitiveness were repeatedly observed by various workers during the subsequent years, as at this time much work was being carried out in guinea-pigs on toxin-antitoxin neutralization. Theobald Smith in 1904, while standardizing diphtheria antitoxin, prepared in the horse, by the inoculation of guinea-pigs, found that these animals, after receiving the horse serum, acquired a marked susceptibility to its subsequent injection. This was termed the *Theobald Smith phenomenon.* This particular reaction has received much attention, and it is mainly as a result of the study of this phenomenon that much of our knowledge of the hypersensitive state has been obtained. The following fundamental features of this reaction have been determined :—

(1) Substances causing hypersensitiveness must be true antigens, *i.e.*, they must be able to stimulate the production of antibodies. The antigens are proteins, which are usually non-toxic and do not produce symptoms in the unsensitized animals.

(2) In the case of antitoxic serum the reaction is due to some substance normally present in the serum and not to the specific antitoxins ; this substance is associated with the serum proteins. Haptenes in the pure state are unable to sensitize, as they do not stimulate the production of antibodies, but as they react with preformed specific antibodies they can induce shock in animals previously sensitized.

(3) The route of the first or sensitizing dose is immaterial ; extremely minute doses, as little as 1 mg. of protein in some cases, may be sufficient to sensitize the animal. If gaining entrance by the alimentary tract the antigen must be absorbed in an intact state. The amount required varies with the nature of the antigen and the type of animal injected ; in all cases it must be sufficient to evoke an antibody response. With certain antigens, such as bacterial proteins, and in some animals, such as the dog, several injections may be necessary to induce hypersensitivity.

(4) Following the sensitizing dose a variable period of time, which is never less than 5 days, elapses before the animals become hypersensitive. This so-called *incubation period* depends upon the type of the antigen and the amount given in the sensitizing dose or doses ; as a rule the smaller the amount the longer the time interval. The ideal time for demonstrating hypersensitivity is that at which minimum amounts of antibody are detectable in the blood-stream and maximum amounts are present in the tissue-cells.

(5) In the case of the "shocking" or assaulting dose, the amount and the route of injection are important factors ; the intravenous route produces a more marked reaction than either the intraperitoneal or subcutaneous route.

(6) The reaction is specific, in that the same or an antigenically related substance is necessary for both the sensitizing and assaulting doses.

(7) Different animals vary considerably in their behaviour, but the same series of symptoms are always exhibited by the same species of animal, irrespective of the nature of the antigenic substance. In all cases anaphylactic shock is a symptom-complex

involving mainly respiratory and vaso-motor action and closely resembles that following the injection of histamine. The guinea-pig is the most susceptible animal ; within a short period after receiving the assaulting dose, the animal becomes restless, convulsions and respiratory distress soon appear and death, which is mainly due to spasm of the bronchial muscles, supervenes. The rabbit is also very susceptible, but larger antigenic doses are required to elicit the response ; a fall in blood pressure usually occurs. Man does not exhibit a high degree of hypersensitiveness and the general effects, when present, are seldom severe.

(8) Once actively sensitized an animal may remain so for a considerable time, in some cases for as long as several years ; the period of marked sensitivity is, however, usually of a relatively short duration, probably a few months.

Passive Anaphylaxis. Anaphylaxis may be passively induced in an individual or animal by the inoculation of the serum of an animal already sensitized or immunized. This hypersensitive state is of short duration and usually lasts only 8–10 weeks. Some time, a few hours up to 6 days, must, however, elapse after the injection of the serum before the recipient can respond to the assaulting dose of the antigen, which must be that employed for the initial sensitization. During this time interval the freshly introduced antibodies are removed from the general circulation and become fixed by the tissue-cells. The simultaneous inoculation of serum from a sensitized animal and the antigen has no effect. The efficacy of a serum to induce passive anaphylaxis has been found to be closely related to its concentration of the specific antibody ; sensitization thus consists essentially in the introduction of antibodies into the host, and an immune serum, which is relatively rich in antibodies, is consequently more efficacious than one from a sensitized animal. Hypersensitivity may be passively induced *in utero* in the offspring of a sensitized mother and lasts about 8–10 weeks.

Desensitization or Anti-anaphylaxis. When animals recover from anaphylactic shock they do not react to further injections of the same antigen for some considerable time. This desensitization or anti-anaphylaxis appears immediately after recovery from the shock. Desensitization may also be produced either by the repeated injection of the sensitized animal subcutaneously with small doses of the antigen or by the injection of comparatively large amounts of the antigen during the so-called " incubation period". Although desensitization is only a temporary measure it constitutes an important practical step in the prevention of unpleasant reactions, particularly during serum therapy. It is important to note that, for the usual method of desensitization, small doses of the antigen must be given, as absorption from the tissues must be slow. It is considered that during desensitization the tissue antibodies are slowly saturated by the antigen and thus become no longer receptive to it.

The Mechanism of the Reaction. Many theories have been advanced to explain the phenomenon of anaphylaxis. Only two, however, merit serious consideration; these are the *humoral* and *cellular* hypotheses. In both it is accepted that an antigen-antibody reaction is involved.

The Humoral Theory has been strongly supported by Friedberger. It holds that, after the union of antigen and antibody, a cleavage of the resulting combination is produced by the action of complement and that this cleavage product, which has been termed *anaphylatoxin*, has toxic properties and is the responsible factor in producing anaphylaxis. This theory is, however, unable to provide a satisfactory answer to the various problems involved in the reaction. It has been found that a number of substances are able to toxify serum *in vitro*, so that on intravenous injection of the guinea-pig anaphylaxis-like or anaphylactoid symptoms are produced; but no direct evidence has been obtained to indicate that an *anaphylatoxin* is produced *in vitro* as a result of antigen-antibody union. Moreover, the short period of time elapsing between the injection of the assaulting dose and the onset of symptoms does not fit in with the suggested mechanism of the humoral theory.

The Cellular Theory postulates that the union of antigen and antibody takes place in or on the body-cells and not in the circulating blood-stream. As a result of this there is a direct stimulation of the unstriped muscle and the capillary endothelium, and this gives rise to the associated symptoms.

The reaction of the tissues has been extensively studied *in vitro* by Dale and others. The uteri of guinea-pigs, sensitized and non-sensitized, have been brought into contact outside the body with the specific antigen; in the case of the sensitized animals the uterus exhibited marked contraction. The reaction of the unstriped muscle does not, however, provide an explanation for all the symptoms. Further research has indicated that certain symptoms may be due to the liberation by the damaged tissue-cells of either histamine or a complex of similar activity. The amount of histamine liberated is largely dependent on the extent of the tissue-damage.

In anaphylaxis it appears therefore that, after the preliminary sensitization, the antibodies disappear from the circulation and become fixed by the tissue-cells. On further inoculation of the antigen, after a certain time limit, union of the antigen and antibody occurs in or on these tissue-cells. Cell injury takes place and histamine is liberated ; the symptoms are thus referable to both the damaged cells and the liberated histamine. There is thus one striking distinction of a quantitative nature between anaphylaxis and immunity, and that is in the concentration of antibodies in the circulation. In immunity the antibodies are present in the circulating blood ; union with antigen therefore takes place in the circulation and unpleasant symptoms do not result. In anaphylaxis the antibodies have disappeared from the blood-stream and tend to be fixed by the tissue-cells, on or in which union occurs, and as a result of this unpleasant symptoms are produced.

Idiosyncrasy

In man many examples of hypersensitiveness are encountered ; these have been termed " idiosyncrasies " by Doerr, " atopies " by Coca and " allergies " by other workers. Idiosyncrasy, however, appears to be the most suitable term, and it is adopted here.

Hypersensitiveness in man does not usually give rise to the serious reactions encountered in the laboratory animals ; there is only a slight tendency to acute generalized shock, but there is a relatively greater tendency to localization in certain tissues. The symptoms thus tend to be localized and their development depends mainly on the route of administration of the antigen, the precise nature of which is not of such great importance, as quite different substances may produce the same symptom-complex. The various clinical manifestations may follow introduction of the antigen—(1) directly into the tissues, as in serum-sickness following serum-therapy, (2) by inhalation into the upper respiratory tract, as in the production of hay-fever and asthma by pollen, dusts, dandruff, face-powders containing orris root, etc., (3) *viâ* the intestinal tract, as seen in urticaria, eczema, asthma and angioneurotic œdema, after the ingestion of certain foods such as strawberries and shell-fish, and (4) *viâ* the skin, as in contact with certain plants. It is now considered that various drug idiosyncrasies may be produced in a similar manner. The drugs themselves are non-antigenic, but they form, by contact with the tissues, compound antigens which are foreign to the body and so are able to stimulate antibody production. The term *atopen* is sometimes given to substances which are non-protein and not true antigens but which are nevertheless able to produce hypersensitiveness.

These reactions are strictly analogous to anaphylaxis and may be produced actively or passively. The production of passive hypersensitiveness can be readily demonstrated by means of the *Prausnitz-Küstner test*: in this the serum of a sensitized individual is injected intradermally into a normal person, some 6–12 hours later the homologous antigen is injected into the same site, and a well-marked urticarial reaction develops. Injections of the antigen into the neighbouring healthy skin is without effect.

The susceptibility of man to the various causative agents may be tested by the intradermal injection of extracts of these substances; hypersensitiveness is indicated by the appearance of a local erythema and œdema. This is an important practical step in identifying the causative agent, as desensitization may subsequently be carried out by a course of graduated doses of the responsible substance. Desensitization is usually a more difficult process in the idiosyncrasies than in anaphylaxis, but it has been practised with some success in the prevention of serum sickness, hay-fever and some cases of asthma; prolonged courses may be required.

In the prophylaxis of hay-fever, an extract of grass-pollen, *Pollaccine*, has given very good results. The sensitivity of the patient is tested either by conjunctival or intracutaneous tests with the extract. A long course of injections is usually necessary as, if too large a dose is administered, very severe reactions may be produced, and consequently it is the usual practice to commence the inoculations in the late winter or early spring and continue with gradually increasing doses at weekly or fortnightly intervals until the pollen season is finished, about the end of July. Under such conditions patients liable to hay-fever generally become desensitized and escape attacks during the same year. It is, however, important to note that the desensitization is not permanent and must be carried out each year.

Similar courses of desensitization are applied to cases of sensitivity to many other substances but the results have been very variable. In some cases satisfactory results have been obtained, while in others little benefit has been derived.

In the case of asthma, desensitization is frequently complicated by the fact that the sensitivity is multiple, and therefore treatment with all substances, with which the patient is likely to come in contact, is necessary.

Heredity appears to play an important part in the development of the hypersensitive state. There is no doubt that hypersensitiveness tends to run in families, but it is interesting to note that the manifestations in the parent and offspring are not necessarily the same. Thus the parent may suffer from hay-fever and the offspring from asthma. This suggests that the hereditary factor is not concerned with the passage of maternal antibodies to the offspring, but rather involves the general sensitivity of the tissues to antigenic stimulation. Further evidence of this is that the inherited tendency to hypersensitiveness may not become apparent until many years after birth. Man comes in contact with innumerable antigens and consequently has limitless opportunities of becoming sensitized. The exact time or times of primary sensitization may, however, be difficult or impossible to determine with any accuracy, but it is often noticed that individuals become sensitized to substances with which they come in close contact. There is thus a relationship between hypersensitiveness in man and anaphylaxis in the experimental animal. This is particularly evidenced in serum sickness, which may be either acquired, *i.e.*, resulting from a definite external stimulation, or natural, in which case there is no history of sensitization. The acquired form is strictly analogous to the phenomenon of anaphylaxis. A second injection of the same foreign serum (usually horse serum) more than 10–14 days after a previous dose may give rise to symptoms which are occasionally dangerous, particularly in asthmatics. This is well recognized in serum-therapy, and great care is taken to avoid such a complication. Under these circumstances desensitization is carried out, usually by the subcutaneous or intramuscular injection of a small dose of the serum. If unpleasant symptoms do not appear the second dose of serum can be given a few hours later. With highly sensitive cases it may be necessary to administer the serum subcutaneously in a series of graduated doses, commencing with an extremely minute dose (0·01 c.c.) and increasing at half-hourly intervals in the absence of symptoms. If symptoms of shock, such as dyspnœa, pallor and collapse, develop they may be relieved by the immediate injection of adrenalin or ephedrine, which relax the muscular spasms.

In certain cases symptoms may follow the first injection of a foreign serum into the tissues after an incubation period usually of less than 14 days. The symptoms usually consist of an urticarial eruption, perhaps with joint-pains. The mechanism of this form of the condition is undoubtedly similar to that of the acquired form and results from the presence in the tissues of antibodies, developed as a result of the injection, acting on the remaining foreign protein of the inoculated serum.

In rare instances when serum is injected into particularly sensitive individuals, such as asthmatics, a severe reaction, which may prove fatal, may appear within a few minutes of the administration of the serum. Dyspnœic symptoms are usually prominent and adrenalin (1/1,000) or ephedrine must be injected either intravenously or intramuscularly without delay. With improved methods of concentrating therapeutic sera and the consequent removal of much extraneous material, the incidence of serum reactions is now relatively low. As, however, these reactions are particularly liable to recur in people with a history of hypersensitiveness, such as asthma or hay-fever, attempts have been made to detect individuals sensitive to serum. Two tests, conjunctival and intracutaneous, have been used but neither has proved entirely satisfactory. In the former, serum diluted ten times in saline is dropped on to the conjunctiva and, if a positive reaction develops, signs—itching, congestion and lachrymation—develop within 15 minutes. In the intracutaneous test, the serum is injected into the skin, and if positive marked erythema develops within a few minutes.

Allergy

A specific hypersensitiveness to products of the infecting organism is encountered during the course of certain bacterial infections, and to this phenomenon the term *allergy* is frequently restricted. The term " allergy " has been employed by different workers to indicate various hypersensitive conditions, but it appears more satisfactory, in view of our restricted knowledge, to limit it to this particular form.

Individuals and animals infected with the tubercle bacillus exhibit an increased sensitivity to an extract of the tubercle bacilli, termed *tuberculin*. On the subcutaneous inoculation of such individuals with tuberculin, reactions occur locally at the site of inoculation, focally at the focus of infection and also generally. This reactivity is frequently associated with an accompanying resistance to reinfection with the tubercle bacillus and is referred to as " Koch's phenomenon " (see Chapter XXIV.). This is applied to the diagnosis of tuberculosis in the form of the Mantoux and von Pirquet tests, in which tuberculin is applied intradermally and cutaneously respectively ; a positive result indicates infection with the tubercle bacillus.

An allergic state is also encountered in other diseases, such as glanders. Only individuals infected with the particular organism react, and consequently skin tests are of assistance in diagnosis, especially in atypical forms of the disease. In pneumonia an allergic reaction can be obtained by the intradermal inoculation of the specific carbohydrate of the homologous pneumococcus. This reaction is utilized as a means of assessing the serum-dosage in the treatment of pneumonia. Some workers also consider that asthma may be a primarily allergic phenomenon resulting from infection of the upper respiratory tract.

While there are definite differences between allergy and anaphylaxis, this does not necessarily indicate that different mechanisms are involved in these two reactions. The reactions are generally more complex, but this may be due to the complex antigenic structure of bacteria. It has been found that each fraction may produce an allergic response, which varies in degree with the different fractions. The bacterial antigen concerned primarily with immunity may therefore not be the one mainly involved in the allergic reaction. It has been found recently that in the case of tuberculosis allergy and immunity are not directly dependent upon each other. In the case of tuberculin, the lesion following the intradermal injection of a tuberculous patient is quite distinct from the skin reaction in protein sensitization, being of an inflammatory nature ; the nature of the responsible antigen has not been determined, but it is closely connected with the bacterial protein. The reaction, however, appears to depend primarily on an antigen-antibody union in the tissues and should be considered as a manifestation of immunity response.

CHAPTER XII

THE CLASSIFICATION OF BACTERIA

Bacterial nomenclature from its early days has been loose and unsystematic; the terms in common use, consequently, have not the significance that applies to terminology in other branches of biology. One of the reasons for this unsatisfactory state is that the identification of certain organisms may be a matter of considerable difficulty, and many criteria must be fulfilled before their identity can be definitely established. It is therefore not surprising to find that many designations were used by different workers in the early days of bacteriology to indicate the same organism; for example, the following names have been given at various times to *Cl. welchii*:—*B. aerogenes capsulatus*, *B. perfringens*, *B. phlegmonis emphysematosæ* and *B. enteritidis sporogenes*. This is obviously very confusing and, although the medical bacteriologist has a limited sphere of activity, the necessity for a generally accepted classification can be readily appreciated.

One of the important tasks confronting the bacteriologists is the clearing up of this chaotic state and the establishment of a uniform nomenclature; with this aim an international committee has recently been appointed. Progress will doubtless be slow, as it is highly probable that medical bacteriologists will continue to use such time-honoured but loose descriptive terms as the tubercle bacillus, diphtheria bacillus and gonococcus, in preference to names of more taxonomic significance.

Many attempts have already been made to classify the bacteria. The early efforts were based mainly on morphological studies but, in view of the inherent fallacies involved, such schemes are now mainly of historic value. The fundamental bacterial forms, viz. coccus, bacterium, vibrio, spirillum and spirochæte, were, however, clearly recognized.

A simple but useful classification has been based on the reaction of bacteria to staining by Gram's method; two primary subgroups are formed—(1) Gram-positive and (2) Gram-negative bacteria. The arrangement of organisms important to the medical bacteriologist is given in Table IV.; it is important to recognize that irregularities, particularly when dealing with old cultures, are not uncommon.

TABLE IV

Reaction to Gram's Stain

Gram-positive	Gram-negative
Staphylococcus.	Gonococcus.
Streptococcus.	Meningococcus.
Pneumococcus.	*N. catarrhalis.*
Anthrax bacillus (*B. anthracis*) and pseudoanthrax bacilli.	Influenza bacillus (*H. influenzæ*).
Diphtheria bacillus (*C. diphtheriæ*) and diphtheroids	*B. pertussis*
Tetanus bacillus (*Cl. tetani*), *Cl. welchii* and other Clostridia.	*Br. abortus* and *melitensis*
Actinomyces.	*V. choleræ.*
Tubercle bacillus (*M. tuberculosis*).	Members of the coli-typhoid-dysentery group.
	Friedlander's bacillus.
	Plague bacillus (*Past pestis*)

Later the importance of other criteria, such as cultural characteristics, biochemical activity and serological reactions, was realized and more comprehensive schemes were suggested. One of the most valuable contributions in this respect was the classification issued by the American Society of Bacteriologists in 1920. While there are many points which have not been generally accepted, this scheme did much to direct attention to the value and importance of a standard system of nomenclature. Further advance was made at the first congress of the International Society for Microbiology held in Paris in 1930, when a Nomenclature Committee was authorized. Progress has already been made by this committee, who have recently defined a new genus *Salmonella* of the tribe *Bactereæ*. This tribe is extremely large and unwieldy, but the various attempts to sub-group the members have been far from satisfactory. Such terms as *Escherichia*, *Salmonella*, *Shigella*, *Aerobacter*, and *Eberthella* have been used to indicate different genera and sub-genera, but no generally accepted scheme has been introduced. These various groups are

TABLE V

Class—Schizomycetes

Order	Family	Tribe	Genus	Remarks
Eubacteriales	Nitrobacteriaceæ			Non-pathogenic to man
	Coccaceæ	Micrococceæ	Micrococcus Sarcina	Usually non-pathogenic
		Streptococceæ	Streptococcus Diplococcus Staphylococcus	Some members highly pathogenic
		Neissereæ	Neisseria	Including the meningococcus and the gonococcus
	Spirillaceæ		Vibrio	Some members, *e.g.*, V. choleræ, pathogenic
			Spirillum	Non-pathogenic to man (generally)
	Pseudomonadaceæ		Pseudomonas	Usually non-pathogenic
	Bacteriaceæ	Bactereæ	Bacterium	Coli-typhoid-dysentery group
			Proteus	
		Chromobactereæ	Chromobacterium	Non-pathogenic
		Lactobacilleæ	Lactobacillus	Usually non-pathogenic
		Hæmophileæ	Hæmophilus	Pathogens, including influenza bacillus
		Pasteurelleæ	Pasteurella	Pathogens, including the plague bacillus
			Brucella	Pathogenic to man and animals
	Bacillaceæ		Bacillus	Usually saprophytes, but including the anthrax bacillus
			Clostridium	Usually saprophytes but some pathogenic *e.g. Cl. tetani.*
Actinomycetales	Mycobacteriaceæ		Mycobacterium	Including the tubercle bacillus
			Corynebacterium	Including the diphtheria bacillus
			Pfeifferella	Including the Glanders bacillus
			Fusiformis	Usually non-pathogenic
	Actinomycetaceæ		Actinomyces	Including A. bovis
			Actinobacillus Erysipelothrix	Animal pathogens
			Leptotrichia	Usually non-pathogenic
Spirochætales				

here included in the genus *Bacterium*. It is admitted that the use of this term in the generic sense has little to commend it and that there is much to support the present tendency to establish different sub-genera or genera. However, until a scheme has been completed by the International Sub-Committee it appears most advisable to accept a *status quo*. The proposed genus *Salmonella* has consequently been considered here only as a sub-group of the genus *Bacterium*.

The system developed by the American Society of Bacteriologists is usually adopted as a basis of most schemes of classification; this practice has been followed, although several amendments have been made, particularly in the generic names of the tribe *Bactereæ*. Many criteria have been employed in formulating this scheme—morphology, cultural characteristics, biochemical activity, antigenic structure and pathogenicity. The use of a single criterion is futile, as any one may exhibit considerable variation.

Bacteria belong to the class " Schizomycetes ", which is further sub-divided into orders, families, tribes, genera and finally species. Under this scheme an organism is given the generic name first, with a capital, and the species name afterwards with a small letter, *e.g.*, *Bacterium coli*, *Clostridium welchii*, *Mycobacterium tuberculosis*, etc. With a knowledge of the generic definitions these terms are considerably more informative to the bacteriologist than the earlier descriptive terms, such as *B. tuberculosis*, *B. influenzæ* and *B. coli*, all of which apparently indicated a similar type of organism. It is, however, unfortunate that the two terms, " bacterium " and " bacillus," which are usually employed in a general sense, should now be used also as generic titles; however, to avoid confusion these terms, when used in the generic sense, are given capitals.

The main features of this scheme are summarized from a medical viewpoint in Table V.; it is important to note that this is merely an abbreviation of the complete classification. Certain orders, families, tribes and a large number of genera of non-medical interest have been omitted.

The " Rickettsiæ " and the " Viruses " have not been included in the table, although there is now much evidence to indicate that the rickettsiæ and many of the viruses are minute forms of bacteria with a highly parasitic existence. Rickettsiæ have a similar morphological appearance to bacteria but they can only be cultivated in media containing living cells. The viruses vary considerably in size from relatively large forms having a definite cell structure, such as the vaccinia and influenza viruses, to certain plant viruses which appear to be merely large molecules composed of nucleoprotein. The large viruses appear to be minute forms of bacteria leading an essentially parasitic and intracellular existence; the taxonomic position of the smaller forms is, however, uncertain, as they seem to be at the limit of living bodies.

The following are the main features of the important genera, together with the name of the type species or a representative member of each :—

Micrococcus. Spherical cells arranged in irregular masses (never in long chains or packets); generally Gram-positive; grow well on agar frequently with the production of a yellowish pigment; usually non-pathogenic to man or animals.

Type species—*Micrococcus luteus.*

Sarcina. Gram-positive spherical cells dividing in three planes producing cubical packets; non-pathogenic to man.

Type species—*Sarcina ventriculi.*

Streptococcus. Gram-positive cocci, arranged in long or short chains; growth tends to be slight on the simpler media; many are parasitic and some are highly pathogenic to man.

Type species—*Streptococcus hæmolyticus.*

Diplococcus. Gram-positive cocci, usually arranged in pairs of somewhat elongated cells; parasites.

Type species—*Diplococcus pneumoniæ* (pneumococcus).

Staphylococcus. Gram-positive cocci, arranged in clusters on solid media, and in pairs, short chains or small groups in fluid media; grow well on artificial media and form pigments; many are parasites and some are highly pathogenic to man.

Type species—*Staphylococcus pyogenes.*

Neisseria. Gram-negative cocci, usually arranged in pairs; growth poor on the simple media; parasites, and some are highly pathogenic to man.

Type species—*Neisseria gonorrhϾ* (gonococcus).

Vibrio. Short, Gram-negative, curved, rigid, motile rods; non-sporing; many are saprophytes, but a few are pathogenic to man.

Type species—*Vibrio choleræ.*

Pseudomonas. Gram-negative, non-sporing rods; usually form a water-soluble greenish or yellowish pigment which diffuses through the medium; commonly found in water and soil.

Type species—*Pseudomonas pyocyanea* (*B. pyocyaneus*).

Bacterium. Gram-negative, non-sporing rods, growing readily on the ordinary media ; with the exception of *Bact. alkaligenes* all ferment glucose with the production of acid, or acid and gas ; most members are intestinal parasites of man and animals, but some are found on plants and in the soil ; many are pathogenic to man.

Type species—*Bacterium coli* (*B. coli*).

Proteus. Gram-negative, motile, non-sporing rods ; actively proteolytic ; form a spreading surface growth on moist agar ; ferment glucose and sucrose (but not lactose) with the formation of acid and gas.

Type species—*Proteus vulgaris.*

Lactobacillus. Gram-positive, non-motile, non-sporing rods ; generally produce lactic acid from carbohydrates ; usually micro-aerophilic ; surface growth on media poor.

Type species—*Lactobacillus caucasicus* (*B. bulgaricus*).

Hæmophilus. Gram-negative, non-motile, non-sporing, minute rods, which, however, may be highly pleomorphic ; require for growth certain accessory factors, which are present in blood, yeast and some plant tissues ; parasites ; some pathogenic to man.

Type species—*Hæmophilus influenzæ* (influenza bacillus).

Pasteurella. Small Gram-negative, non-sporing rods showing bipolar staining ; limited biochemical activity ; parasites ; pathogenic to man and animals.

Representative species—*Pasteurella pestis* (plague bacillus).

Brucella. Gram-negative, non-sporing cocco-bacilli ; aerobic or micro-aerophilic ; fail to ferment carbohydrates ; strict parasites ; pathogenic in man and animals.

Type species—*Brucella melitensis.*

Bacillus. Aerobic, Gram-positive, spore-bearing rods ; generally liquefy gelatin ; usually saprophytes ; one member (*B. anthracis*) highly pathogenic to man.

Type species—*Bacillus subtilis.*

Clostridium. Gram-positive, anaerobic or micro-aerophilic rods, forming spores, which are wider than the vegetative form ; some species pathogenic to man.

Representative species—*Clostridium welchii* (*B. welchii*).

Mycobacterium. Slender, non-motile, non-sporing rods, which stain with difficulty, but, when once stained, are acid-fast ; aerobic ; growth on artificial media slow ; some species are highly pathogenic to man and animals.

Type species—*Mycobacterium tuberculosis* (tubercle bacillus).

Corynebacterium. Gram-positive, non-motile, non-sporing rods often showing characteristic arrangements and irregular staining reactions; aerobic; some pathogenic species produce a powerful exotoxin.

Type species—*Corynebacterium diphtheriæ* (diphtheria bacillus).

Pfeifferella. Slender, Gram-negative, non-motile, non-sporing rods; growth slow; characteristic brownish growth on potato.

Type species—*Pfeifferella mallei* (Glanders bacillus).

Fusiformis. Non-sporing, non-motile rods, which stain unevenly and grow poorly on artificial media.

Representative species—*Fusiformis dentium* (*B. fusiformis*).

Actinomyces. Non-motile organisms growing in the form of a branched mycelium, which may break into fragments; some species are micro-aerophilic or anaerobic, and some are acid-fast; some pathogenic species form radiating threads with clubbed ends in the tissues.

Type species—*Actinomyces bovis.*

Leptotrichia. Thick, long, Gram-positive, non-motile unbranched threads, which may fragment into short rods; anaerobic.

Type species—*Leptotrichia buccalis.*

Spirochætales. Protozoan-like in many characters; cells consist of slender flexuous spirals; multiplication by transverse division; no flagella.

CHAPTER XIII

BACTERIOLOGY AND MEDICINE

In view of the importance of bacteria as disease-agents in man, it is not surprising to find that the application of various bacteriological methods to the diagnosis, prevention and treatment of disease has yielded valuable and far-reaching results. The striking developments in public health practice during the past half-century have been largely influenced by the remarkable increase in the knowledge of bacteria during this period. Investigations into the mode of infection in various diseases and the *rôle* played by insects and carriers have had remarkable results. Such diseases as rabies, typhus fever, cholera, small-pox and plague have been practically eliminated from this country, while the incidence of certain others, *e.g.*, anthrax and the enteric fevers, has been greatly reduced. In many essentially human diseases, particularly those of the upper respiratory tract, it is impossible to control or even to reduce at all adequately the risks of infection. In such cases the aim is consequently to increase the resistance of the community by specific methods, so that infection is either prevented or reduced in severity.

The application of bacteriology to medicine, in so far as the medical practitioner is concerned, may be conveniently considered in five main groups :—

(1) The identification of pathogenic bacteria.
(2) The immunological diagnosis of disease.
(3) The detection of individuals susceptible to a given disease.
(4) Specific prophylaxis and therapy.
(5) The control of chemotherapy.

1. The Identification of Pathogenic Bacteria. The isolation of pure cultures and the subsequent identification of the particular organism are problems of fundamental importance, as they constitute factors which are essential for the control of many infectious diseases and also for the application of rational serum and chemo-therapy.

The incrimination of a particular organism in a disease-process may be a matter of considerable difficulty. The surfaces of the body harbour many organisms, which may interfere with the isolation of that responsible for the lesion. Complications may also arise from the presence of mixed, secondary or terminal infections. In the case of many easily recognized infections, such as diphtheria and gonorrhœa, the presence of the responsible organisms in the lesions provides the bacteriologist with sufficient evidence to make a diagnosis. On the other hand, in examining a disease of unknown origin it is necessary to exercise much caution before incriminating a particular organism. The suspected organism must be isolated in pure culture and, after identification, the fulfilment of Koch's postulates should be attempted.

The difficulties involved in some cases may be illustrated by the case of influenza. Pfeiffer, some 50 years ago, described the influenza bacillus and stated that it was the ætiological agent of influenza ; it is only in recent years that a virus has been proved the causative agent of this disease, while the influenza virus plays the *rôle* of a secondary invader.

Some organisms can be readily identified but, in other cases, considerable difficulty may be experienced and the examination may last for several weeks. There is thus no simple method of general application whereby an organism can be identified. The following scheme is recommended for general application as it ensures a systematic approach to the question ; in some instances only a few of the examinations may be required, but in others all or most must be completed before the identity of an organism can be established.

(*a*) A consideration of the nature and source of the material with the history of the case.

(*b*) Morphological and staining characteristics.

(*c*) Cultural methods.

(*d*) Biochemical reactions.

(*e*) Serological tests.

(*f*) Animal inoculation.

(*a*) In all cases a detailed account of the material, together with a clinical history of the case, should be available. Absence of these may frequently cause much unnecessary labour and expense, as the source and nature of the material generally suggest the line of attack. Thus urethral pus may require methods of examination quite different from those employed in the examination of pus from cases of empyema, meningitis or a subcutaneous abscess. A naked-eye examination of the material frequently provides useful information of the type of infection present. Fluid fæces with hæmorrhage suggests dysentery; the presence of yellowish granules in samples of pus indicates actinomycosis; a relatively clear pleural or cerebro-spinal fluid directs attention to the possibility of infection with the tubercle bacillus. The presence of a foul odour is usually indicative of the activity of some proteolytic organism, such as the anaerobic bacteria.

The **collection** of the various samples also requires close attention. All risk of contamination must be avoided, and special methods are used in obtaining different specimens. When collecting blood or material, such as pus, from the subcutaneous tissues or serous cavities, the skin is treated with some disinfectant, such as iodine, and the material is collected by means of a sterile syringe. Blood is usually obtained from the median vein in the antecubital fossa. In the case of superficial lesions, such as skin abscesses or urethral pus, the local lesion is well cleansed, and fresh material, *e.g.*, pus or vesicular fluid, is expressed and collected either in a sterile pipette or on a sterile swab or the platinum loop. Suitable media, such as blood-agar or broth, are then inoculated.

The collection of urine for a general bacteriological examination is carried out by means of a catheter into a sterile container. In the case of males a mid-stream specimen is often satisfactory.

In the case of throat swabs the patient's mouth should be opened as widely as possible, the tongue depressed with a spatula and the patient asked to say "ah-ah." Illumination should be provided by a pocket-torch and the swab rubbed firmly against the lesion; it is important to avoid touching the healthy mucosa of the mouth. Material from the naso-pharynx is collected on a West's swab, which is a long flexible wire with a swab at one end; this is contained in a curved glass-tube which is plugged at both ends. When required the plugs are removed, the tube held at the back of the mouth, the curved portion being directed towards the naso-pharynx and the swab is pushed out and rubbed against the mucosa; it is then replaced in the glass-tube and withdrawn. The swab is protected by the glass-tube and unnecessary oral contamination is thus avoided.

Cerebro-spinal fluid is obtained by either lumbar or cisternal puncture. For lumbar puncture the patient should be in a horizontal position with the knees drawn up to the chin. After thoroughly cleaning the skin with spirits, lysol or iodine puncture is made between either the 2nd and 3rd or 3rd and 4th lumbar spines, the local skin and subcutaneous tissue being first anæsthetized with novocain. When the spinal canal is reached the stylet is withdrawn ; the fluid is then collected in a sterile tube. About 5–6 c.c. are normally removed, but in cases of acute meningitis the quantity should be much greater.

(*b*) After these preliminary observations microscopic examination is carried out in order to determine the morphological and staining reactions of any bacteria that may be present in the sample. When the material is a relatively clear fluid, *e.g.*, urine or fluid fæces, an unstained preparation is examined. By this means the form of the organisms and the absence or presence of motility can be detected, while the type of the accompanying tissue-cells is also indicated. In dealing with certain serous exudates, such as fluid from primary chancres, dark ground-illumination is used ; in the case of chancre-fluids, if the *Trep. pallidum* is observed, further examination of the material is not required in a routine examination. Stained films are, however, necessary in practically all other cases, one notable exception being the examination of fæces for members of the typhoid-dysentery group of bacteria. Gram's method is carried out as a routine procedure, but, where indicated, special methods are also employed, as in the examination for diphtheria or tubercle bacilli. Stained smears yield valuable results and, in a few cases, they may be solely relied upon to identify an organism, *e.g.*, the gonococcus in the urethral pus from acute cases of gonorrhœa, tubercle bacilli in sputum and rarely diphtheria bacilli in throat swabs. In the majority of instances identification is impossible by microscopy alone, but sufficient information is obtained to indicate media suitable for cultural purposes.

(*c*) The combination of cultural and morphological examinations is sufficient to establish the identity of many organisms. Agar, broth and blood-agar are usually employed in routine practice, but in some cases selective and inhibitory media give the most satisfactory results. For example, tellurite media are of great value in the isolation of the diphtheria bacillus from throat swabs, while the desoxycholate-citrate medium is now widely used in the examination of fæces for the intestinal pathogens. With solid media the colonial appearance, pigment formation and the presence of any alteration in the medium, *e.g.*, hæmolysis, are points of interest ; in fluid media attention is directed to the formation of a pellicle, deposit or turbidity.

(*d*) In some cases it is necessary to utilize biochemical tests in order to differentiate closely related species. Fermentation tests are especially useful; 0·5–1 per cent. of the various sugars and a suitable indicator are added to peptone-water or Hiss's serum-water. Fermentation is indicated by the production of acid with or without the formation of gas. The behaviour of certain organisms is remarkably constant and the tests are used as a basis of sub-grouping. Many carbohydrates are used in the identification of the intestinal bacteria, which are primarily divided into two groups—the lactose-fermenters (the non-pathogenic members) and the non-lactose-fermenters (the pathogenic members); glucose and maltose serve to differentiate the gonococcus from the meningococcus, while inulin may be of value in the separation of the pneumococcus from *Strept. viridans*. Proteolytic tests are also extensively applied in the identification of bacteria; the usual methods of examination are the production of indole, the digestion of meat and the liquefaction of gelatin and serum.

(*e*) Further investigations may be required before the identity of an organism can be definitely established and it is then necessary to carry out serological tests. Agglutination, precipitation and complement fixation are used, and of these agglutination usually gives the most satisfactory results. The organism is tested against known antisera, which are indicated by the previous examinations. Serology is essential for the identification of the pathogenic members of the *Bacterium* genus; in some cases it is even necessary to use agglutinin-absorption or specific sera to differentiate closely related species such as *Bact. paratyphosum B.* and *Bact. aertrycke.* In pneumonia the typing of the responsible pneumococcus by agglutination or precipitation is necessary for the correct application of serum-therapy.

(*f*) Certain organisms are sometimes most readily isolated and identified by the inoculation of susceptible animals. This procedure is adopted in isolating tubercle bacilli from contaminated material, such as milk or fæces, and the anthrax bacillus from shaving brushes; in the former case guinea-pigs are used, and in the latter either mice or guinea-pigs. Animal inoculation is also used as the final test in the separation of a pathogenic organism from closely related but non-pathogenic species. Thus virulence tests in guinea-pigs are used to distinguish the virulent diphtheria bacillus from avirulent species or the diphtheroids, and the injection of mice or guinea-pigs is usually the final test to differentiate the anthrax bacillus from the pseudo-anthrax bacilli. Laboratory animals are necessary for the isolation of viruses.

It follows from this brief review that the isolation and identification of an organism not infrequently present a complicated task. In some instances the examination may be completed in 24 hours, whereas in others at least 7 days are required. An early result is usually desired by the clinician, and a tentative progress report should always be given whenever a lengthy examination is probable.

2. The Immunological Diagnosis of Disease. In some cases when the isolation of the infecting organism is either extremely difficult or impossible, useful information of the infective process may be obtained by various immunological reactions in the form of serological or skin tests.

Serological tests have been employed for this purpose for many years with much success ; these reactions involve some antigen-antibody union, and if one of these factors is unknown the other can be determined. Two main methods of testing are available, unknown antigens may be tested against known sera and *vice versâ*. The test, in which the antigen is the unknown factor, has sometimes proved useful as a means of typing the responsible organism in cases of pneumococcal pneumonia ; urine or extracts of the sputum frequently give a precipitate with the homologous antiserum.

The other method, *i.e.*, one in which the serum is the unknown factor, is, however, the one most widely applied. Important examples are the Wassermann test in syphilis, complement-fixation test in chronic gonorrhœa, and agglutination tests in enteric fever, typhus fever and undulant fever.

The diagnostic application of the skin test is becoming more widely employed. The substance employed may be either an extract of the agent considered to be responsible for the condition or an immune serum. Examples of the first type of reaction are the allergic tests, such as the tuberculin test in tuberculosis, the abortin test in undulant fever and the mallein test in glanders, and the various intradermal tests for the detection of hypersensitiveness to various proteins, such as pollens and dust. An example of the second type is the Schultz-Charlton reaction in scarlet fever, in which local blanching of the erythematous rash is produced by the intradermal injection of convalescent serum.

3. The Detection of Susceptibles. An important part of preventive medicine is the detection of individuals susceptible to a particular disease. Skin tests have recently proved of great value in differentiating susceptibles from immunes in diphtheria and scarlet fever, these are known as the Schick test and the Dick test respectively. Small doses of the specific toxin are injected into the skin, and if the corresponding antitoxins are absent or present in very small and insignificant amounts an erythematous patch is produced at the site of injection. These reactors are considered to be susceptible to infection with the particular organism and may be rendered resistant by active immunization with the modified toxin, *i.e.*, the toxoid.

4. Specific Prophylaxis and Therapy. The various methods of increasing the resistance of the body to infection by specific methods have already been considered. They may be applied either prophylactically or therapeutically, for both of which active or passive measures may be adopted.

In *prophylaxis*, active immunization is the more valuable procedure as the effects last much longer than those produced by passive methods. The most important practical applications are vaccination against small-pox with calf-lymph or culture-virus, T.A.B. vaccines in the prevention of enteric fevers, the various toxoid mixtures in diphtheria, toxoid in tetanus, attenuated virus in rabies, killed vaccine in typhus, attenuated virus in yellow fever, and the use of convalescent serum some days after infection or exposure to attenuate the resulting infection with the measles virus.

Passive immunization is effective for a short time only, and its application is consequently rather restricted. It is used mainly as a temporary measure to protect delicate children who have, or are likely to, come in contact with cases or carriers of diphtheria, scarlet fever and measles.

In *therapy*, active measures are sometimes useful, as in vaccine and toxoid therapy of boils and abscesses, gonorrhœa, and coliform infections of the urinary tract.

Passive measures have a wider application, as the value of serum therapy in certain conditions is now well recognized. It is, however, important that the potency of the serum should be of a satisfactory standard and that the serum should be administered at the earliest possible opportunity and in adequate dosage. Failure to observe these points has been responsible for many disappointing results, and in order to obviate the former possibility, official standards for those sera of established value were fixed by a committee appointed by the League of Nations.

Sera are of three types—antitoxic, antibacterial and antiviral. Antitoxic sera yield the best results and are extensively used in the treatment of diphtheria, gas gangrene, tetanus, and scarlet fever. Valuable antibacterial sera are much more difficult to obtain, but they have been prepared in a satisfactory manner against the pneumococcus (type I.) and the meningococcus. Serum-therapy was an essential feature of the treatment of certain types of lobar pneumonia and meningococcal meningitis until the recent introduction of the sulphonamide preparations, but it is now seldom necessary. The therapeutic effect of antiviral sera is uncertain, but convalescent and/or immune horse serum have been used with a certain degree of success in the treatment of poliomyelitis, measles and yellow fever.

5. The Control of Chemotherapy. Chemotherapeutic agents, in particular penicillin and the sulphonamides, are now widely used. It is therefore important to appreciate that the action of these substances on different organisms varies considerably, *e.g.*, *Staph. pyogenes* is sensitive to penicillin but relatively insensitive to the sulphonamides. It is consequently necessary that the use of chemotherapeutic agents should be subjected to strict bacteriological control to ensure that an active preparation is given. The sensitivity of bacteria to these substances can be readily determined in the laboratory (cf. Chapter VII.).

PART II

SYSTEMATIC BACTERIOLOGY

CHAPTER XIV

STAPHYLOCOCCUS, MICROCOCCUS AND SARCINA

Staphylococcus

The genus *Staphylococcus* contains Gram-positive cocci that tend to be arranged in small groups or clusters, that show pigmented colonies on solid media and that may be pathogenic to man.

Classification. Bacteria bearing a close resemblance to staphylococci were observed by Koch in 1878, and were first cultivated, in a fluid medium, by Pasteur in 1880. These organisms were grown in eggs and their relationship to abscesses described by Ogston in 1881–82. It was Rosenbach, however, who, in 1884, made a detailed study of them and suggested a classification according to the pigmentation of the colonies : *Staph. pyogenes aureus* and *Staph. pyogenes albus*. In the following year, 1885, Passet added a further variety, *Staph. pyogenes citreus*, which produced a yellow pigment. Since then numerous varieties have been described and given special descriptive names. In many instances, however, these organisms have not been staphylococci, but members of the closely allied *Micrococcus* genus ; in other cases they have probably been varieties of one of the already described species.

Staphylococci have thus been divided into three main subgroups. The criterion on which this classification has been based is, however, far from satisfactory. It has already been mentioned that the formation of pigment by a bacterium is not constant, but is dependent on a number of variable factors, and that it is frequently difficult to decide the type of pigmentation produced by growth for 24 hours on the usual laboratory media. Other methods of classification had, however, proved less satisfactory.

In recent years much work has been carried out on the subject and a new method of classification has been introduced. It has been found that there is a close relationship between the pathogenicity of strains for man and rabbits and their power to form coagulase, *i.e.*, the power to coagulate plasma ; pathogenic strains are coagulase-positive. It has also been shown that coagulase-positive strains generally produce the α toxin, ferment mannite and liquefy gelatin. They embrace most of the strains producing the *aureus* pigment and also *albus* strains causing lesions in man. *Citreus* strains are rarely, if ever, coagulase-positive.

As a result of these observations, made independently by several workers, it is recommended that the term, *Staphylococcus pyogenes*, should be used to indicate potentially pathogenic strains, *i.e.*, those forming coagulase. No satisfactory term has, however, been given to the coagulase-negative organisms. This is unfortunate as the use of pigment-production as a criterion of classification for some strains and not for others is confusing. It has been suggested that *Staphylococcus saprophyticus* would be a suitable designation for these strains.

Further subdivision of the *Staphylococcus* genus may be carried out either by pigment production or in the case of *Staph. pyogenes* by slide-agglutination. Neither of these methods is, however, entirely satisfactory.

Habitat. *Staphylococci* are ubiquitous. They are normally present on the skin and in the alimentary tract of man and animals, in which sites they are frequently responsible for pyogenic lesions ; they are found also in soil, the air, sewage, milk, water, and most articles in use by man. Their natural habitat is considered to be the animal body.

Morphology and Staining Reactions. The *Staphylococci* are spherical bodies with an average diameter of 0·7–1·2μ. The size varies not only of the different species, but also of the members of the same species, according to the age of the culture and the medium in which the growth is taking place. The cocci tend to be larger in old cultures. It is frequently stated that *albus* strains are larger than *aureus* strains.

The name *Staphylococcus* was originally given to this group on account of the tendency of the cocci to be arranged in grape-like clusters. It should be noted that this characteristic grouping is not a constant morphological feature of the *Staphylococcus*. It is usually found when multiplication has taken place on solid media, but in fluid media such as broth, the organisms are not infrequently arranged in pairs, short chains of three or four, small groups or as single cocci. Under such circumstances they may present a similar morphological picture to that given by some strains of *Streptococcus*.

The *Staphylococci* do not possess flagella and are therefore non-motile; they are also non-capsulated and do not form spores.

All members are readily stained by the ordinary aniline dyes. They retain the stain in Gram's method and are consequently Gram-positive. In old cultures or when phagocytosed some cocci may be Gram-negative.

Cultural Reactions. The *Staphylococci* grow readily on the usual media, and in most cases within a wide range of temperature, 12°–43° C.; the optimum temperature for most strains is about 37° C. They multiply under both aerobic and anaerobic conditions, being facultative anaerobes, and prefer a slightly alkaline medium, the optimum reaction of which is between pH 7·4 and 7·6.

In *fluid media*, such as broth, growth is rapid; in 24 hours there is a marked general turbidity with a variable light deposit, which easily disperses on shaking. After further incubation a ring growth at the surface often appears. The characteristic pigment formation is not found.

On *solid media*, such as agar, a heavy growth soon forms. The individual colonies are circular, moist, opaque, smooth, shining and easily picked off; pigment formation is an important feature of these organisms. On fresh isolation *Staphylococci* produce three main types of pigment; that of the *aureus* strains is golden, that of *albus* strains white, and that of *citreus* strains lemon-yellow. The formation of pigment is governed by certain cultural conditions, such as the presence of free oxygen, favourable media, *e.g.*, agar and potato, and a suitable temperature, 22° C. being better than 37° C. On prolonged artificial cultivation pigment-formation becomes considerably reduced and may eventually be lost.

When grown on blood-agar hæmolysis is frequently observed. Although it is recognized that pathogenic strains of *Staphylococcus* form soluble hæmolysins for rabbit and sheep cells, it is doubtful whether hæmolysin production on a blood-agar plate is a true index of pathogenicity. Pathogenic strains do not generally form the hæmolysins under ordinary aerobic conditions, incubation in an atmosphere containing 20–25 per cent. CO_2 is required. Moreover, it is not unusual for strains of *Staph. pyogenes* to show no hæmolysis on plate cultures, while saprophytic strains may do so.

Resistance. The *Staphylococci* are easily killed by destructive agents ; they are destroyed by heat at 60° C. in ½–1 hour and 1 per cent. lysol in 10 minutes. They are particularly sensitive to the aniline dyes, such as gentian-violet, crystal-violet and malachite-green.

They show a marked resistance to low temperatures, not being killed by liquid air, and to drying. Cultures retain their viability after conservation at room-temperature for many weeks.

Biochemical Activity. The fermentation of sugars by the *Staphylococci* is very variable and, although attempts have been made to utilize this property as a means of classification, they have not been very fruitful. Lactose, glucose, maltose, sucrose and mannite are frequently fermented with the production of acid, but not gas. A point sometimes utilized in identification is that *Staph. pyogenes* strains tend to ferment mannite, while most other strains do not. In litmus milk acid and clot are generally produced, and in some cases peptonization may follow.

Variable results are again encountered in the proteolytic activity of these organisms. Indole is never produced, but gelatin liquefaction is frequently found ; in a stab-culture liquefaction begins around the stab and a funnel-shaped liquefied area develops. In this respect the pathogenic cocci tend to be more active than the non-pathogenic members. Thus *Staph. pyogenes* strains liquefy gelatin more frequently than other strains.

Serology. The various serological tests have proved of little value in the identification and classification of the *Staphylococcus*. Agglutination and precipitation tests have received some attention, and the results indicate that pathogenic strains form a group which can be distinguished from non-pathogenic strains.

Slide-agglutination with sera prepared in rabbits has recently been used, and it is claimed that three main serological types of *Staph. pyogenes* can be identified. Irregular results are not infrequently obtained and further work is required before slide-agglutination can be accepted as a routine method of subdivision for *Staph. pyogenes* strains.

The opsonic index estimation was employed at one time to detect the response to vaccine-therapy. The results were frequently inaccurate and the test has not proved very satisfactory.

Toxin Production. During recent years the production of toxin by the *Staphylococcus* has attracted much attention.

The earlier scattered references indicated quite definitely that various toxic substances were produced by the staphylococcus, but the results obtained by different workers were disjointed and irregular. The following products had been described by workers at various times : leucocidin, hæmolysin, dermotoxin, lethal toxin, enterotoxin, nephrotoxin and staphylocoagulase (causing the clotting of plasma). As these were scattered observations, they had been neglected and forgotten until recent years, when their significance has been subjected to close investigation. Attention was particularly directed to this question when it was found that the staphylococcus was responsible for the disaster at Bundaberg in Australia, in which several fatalities followed the subcutaneous injection of toxin-antitoxin mixtures as a prophylactic measure against diphtheria.

Parker, in 1924, demonstrated that organisms, grown in buffered broth containing " proteose-peptone " and incubated for 6 days at 37° C. in an atmosphere of 10 per cent. CO_2, produced a filtrate that was hæmolytic and gave rise to local necrosis on intradermal injection into rabbits ; intravenous inoculations, however, had no effect. These observations were later elaborated by Burnet (1928) in an extensive investigation of the question, as a result of which the clinical importance of the toxin became apparent.

Burnet found that filtrates of *Staph. aureus*, grown under suitable conditions, had three definite actions : (*a*) hæmolytic, (*b*) necrotic on intradermal inoculation into rabbits, (*c*) lethal on intravenous injection into rabbits. These actions were stated to be due to one substance which had the properties of an " exotoxin." Previous work had suggested that different substances were responsible for the various actions, but Burnet found that any difference in these three actions was of a quantitative and not qualitative nature. The lethal action was the first to be inactivated, whereas the hæmolysin was the most active and persistent. The production of a leucocidin, which was demonstrated by Van de Velde in 1894, was mentioned, but not investigated in detail. This substance appeared to be distinct from the main toxin. Burnet also pointed out that there were probably present in the filtrate other factors, which had more action on the human than the rabbit tissues.

These findings have been confirmed repeatedly and this substance is labelled α toxin, and it has been found that toxin production depends largely on cultural conditions. A satisfactory medium is prepared by diluting 2 per cent. agar with 4 per cent.

proteose-peptone broth to give a final strength of 0·89 per cent. agar. When seeded it is incubated at 37° C. in an atmosphere containing 25 per cent. CO_2 for periods up to four days ; the CO_2 is renewed daily and apparently acts as a buffer. The advantage of agar over broth is that it acts as an adsorbent and removes from the broth some unidentified substance which interferes with toxin-formation.

Recent work has indicated that other distinct toxins may be produced by pathogenic strains of the *Staphylococcus*, as pointed out by the earlier observers. Panton and Valentine (1932), on clinical and experimental evidence, stated that the *leucocidin* which destroys human leucocytes is distinct qualitatively from the toxin producing hæmolysis, necrosis and a lethal action. They claim, with some justification, that the leucocidin is an important factor in the pathogenicity of staphylococci for man.

Jordan and others have found that some strains produce an *enterotoxin*, which on ingestion gives rise to symptoms of gastroenteritis in man ; these strains are considered to be responsible for some cases of food-poisoning in man.

Glenny and Stevens (1935) have separated a further toxin, termed beta (β) which varied in amount with the different strains under examination. The β toxin gives the hot-cold phenomenon with sheep corpuscles, *i.e.*, hæmolysis is only slight at 37° C., but is marked on cooling. It is non-hæmolytic for rabbit cells and is not lethal for rabbits except in large doses (*cf.* α toxin).

The nature of *coagulase* has not been determined. It does not appear to be a true toxin as it is non-antigenic. It resembles an enzyme in many respects. It has been suggested that the production of coagulase by *Staph. pyogenes* is largely responsible for the localized type of lesion frequently caused by this organism.

A staphylococcal filtrate may thus exert several toxic actions, some of which appear to be produced by separate and distinct toxins. In most cases one well-defined toxin, *alpha*, is produced and this at present is the one considered to be of greatest clinical significance. There is, however, increasing evidence to indicate that the leucocidin also plays a prominent *rôle* in human infections. The leucocidin has a destructive action in both rabbit and human leucocytes, and is distinct from the α toxin, which has a destructive action only on rabbit leucocytes. The other factors have so far received relatively little attention, but their presence must not be ignored.

Toxin formation has been obtained most frequently with the *aureus* strains and never with *citreus* strains.

Pathogenicity. The *Staphylococcus* is an important human pathogen. Its two important offensive weapons are pyogenicity and toxicity. These properties are not, however, possessed by all strains; the severity of staphylococcal infections is doubtless dependent on the presence of one or both of these factors. Pyogenicity appears to be the more constant property, but the substance responsible for this has not been determined; it appears to be distinct from the toxin.

Staph. pyogenes strains are pathogenic and are responsible for many severe pyogenic infections of man and animals. In man they are frequently the causative agent of skin lesions, such as sycosis, carbuncles and impetigo contagiosa; they are also responsible for cases of empyema, osteomyelitis, septicæmia, perirenal abscess, peritonitis, otitis media, etc. They are frequently present as secondary invaders, particularly of infections of the upper respiratory tract. In the most severe forms a generalization of the infection with septicæmia or pyæmia may result. It is usually found that strains isolated from severe lesions form the *aureus* pigment, while *citreus* strains are rarely, if ever, pathogenic. When a suppurative phlebitis, followed by thrombosis, occurs at the site of the primary infection, the thrombus breaks down and is disseminated by the blood-stream; the particles become impacted in the small vessels of various tissues and local abscesses result. This condition is known as *pyæmia.*

In some cases of food-poisoning *Staphylococcus pyogenes* has been incriminated; proof of this association is frequently difficult to obtain. It has been found experimentally that some strains form an enterotoxin capable of producing gastro-enteritis in man.

The common laboratory animals are relatively resistant to the *Staphylococcus pyogenes*, the rabbit being the most susceptible. In these animals subcutaneous inoculation usually produces a localized abscess. Intraperitoneal and intravenous inoculations are usually fatal; septicæmia, pyæmia and sometimes osteomyelitis develop.

Diagnosis. The staphylococci are readily identified by their morphological characters, staining reactions in smear preparations and cultural reactions on the ordinary media. Growth occurs readily on the ordinary media; the colonies are pigmented, but the precise colour of the pigment is often difficult to distinguish in young cultures.

In order to determine whether a strain is pathogenic, a *coagulase test* may be carried out by either the tube or slide method. In the tube test, human plasma is diluted two to four times with saline and is then inoculated heavily with the organism ; a known positive control organism should also be tested. The tubes are incubated at 37° C. ; coagulation, in positive cases, occurs in 2–5 hours. The " slide " technique may also be used ; the organism is mixed on the slide with a drop of plasma ; if positive, coagulation of the plasma readily occurs. This test is not as reliable as the tube method.

Strains producing a coagulase usually form the *aureus* pigment, ferment mannite and liquefy gelatin and are pathogenic to rabbits ; they have been given the name *Staphylococcus pyogenes.*

The non-coagulase forming strains are considered to be non-pathogenic and are generally *albus* or *citreus* strains ; these have not been given a special name, but the term *Staphylococcus saprophyticus* has been suggested.

Specific Therapy. The milder staphylococcal lesions, such as boils and acne, have provided a fruitful field for vaccine therapy. Vaccines, either autogenous or stock, are prepared from a saline suspension of a 24-hour culture on agar or the deposit of a broth culture ; they are usually standardized to contain 1,000 million cocci per cubic centimetre. The initial dose is 0·05–0·1 c.c., and this is increased, provided no marked reaction occurs, every 7–14 days to a maximum dose of 1 c.c. Severe lesions, such as carbuncles and osteomyelitis, have not responded well to vaccine-therapy.

In view of the recent development in our knowledge of the staphylococcal toxin, the injection of regulated intramuscular doses of the α toxoid has been tried. The number of injections required varies, but eight gradually increasing doses are considered to be an average. A local reaction is often produced, but the clinical results are sometimes good, especially in the case of recurrent furunculosis, styes and carbuncles. Improvement of the lesions may be associated with an increase in the staphylococcal antitoxin content of the serum as tested by hæmolysis-titration with rabbit corpuscles. Estimations of the hæmolytic titre are, however, considered to be of little prognostic value, as lesions may occur in individuals with a relatively high anti-hæmolysin titre.

The α toxoid is prepared by adding 0·1–0·15 per cent. formalin to a toxin of proved potency and incubating at 37° C. for about 14 days. There is as yet no official standard of the antigenic potency of the staphylococcal formol-toxoid.

There has been an increasing tendency to neglect antibacterial factors, which were previously believed essential, and to involve the toxin solely in immunization. This does not appear justifiable and many workers now consider that both antibacterial and antitoxic factors are concerned with the immunity reaction. The use of an autogenous vaccine together with the toxoid is therefore recommended.

In the very severe forms of staphylococcal infection associated with symptoms of toxæmia, antitoxin therapy is recommended. The α antitoxin is prepared in the horse by repeated inoculations of a suitable toxoid, and is standardized against the official dried standard serum by its neutralizing effect on any one of the three activities of the main toxin, viz., hæmolysis, skin-necrosis and lethal effect on intravenous inoculation ; the various tests have given similar results. It would appear, however, that the most satisfactory form of serum would be one prepared against all the toxins, particularly the α toxin and leucocidin.

Chemotherapy. The sulphonamides have given disappointing results in the treatment of staphylococcal lesions. In contrast, penicillin has proved extremely valuable and should be given in all severe types of infection. Dramatic results are often obtained by a routine course of intramuscular injections, such as 20,000 units at three-hourly intervals. Penicillin ointments have proved useful for local lesions such as boils.

Micrococcus

The *Micrococci* are Gram-positive cocci, frequently forming pigmented colonies and having an optimum temperature of 22°–25° C. One member, *M. tetragenus*, has some interesting characteristics. It was first isolated as a secondary invader in pulmonary tuberculosis. The morphological picture is distinctive ; it divides in two planes at right angles and forms *tetrads*, it also forms a well-marked capsule in the animal tissues. The typical appearance may not be found after prolonged cultivation on artificial media. While of extremely low pathogenicity for man, it is highly pathogenic for white mice ; intraperitoneal inoculation of these animals leads to death in 1–3 days.

A considerable number of micrococci exist ; these are non-pathogenic to man, but as they may sometimes be found as secondary invaders or contaminants of skin and naso-pharyngeal infections their differentiation from the *Staphylococcus* and the *Streptococcus* is therefore important.

Sarcina

The *Sarcinæ* are frequent air contaminants. They are Gram-positive cocci, dividing in three planes at right angles, as a result of which they form cubes. On solid media the colonies are usually large and pigmented bright yellow. They are non-pathogenic to man.

CHAPTER XV

STREPTOCOCCUS

Streptococci are Gram-positive cocci that divide in one plane only and so are arranged in chains of varying lengths. Some strains grow poorly on simple media and may be highly pathogenic to man and animals.

Classification. Although cocci bearing a close resemblance to streptococci had been described by earlier workers, the first systematic study of the organism and its pathogenic properties was made by Rosenbach in 1884. As the organism under examination tended to grow in chains and was also isolated from suppurative lesions in man, he gave it the name *Streptococcus pyogenes.* Subsequently streptococci were isolated from a large number of lesions, both in man and animals. Voluminous literature dealing with this organism has accumulated, but unfortunately a considerable portion of this is of a controversial nature. There is, however, no doubt that much progress has been made but some time must elapse before the various problems presented by the streptococci are finally elucidated.

The first attempts at classification of the streptococci were based on morphological findings. v. Lingelsheim described two types : (1) *Strept. longus*, which formed long chains and was usually pathogenic, and (2) *Strept. brevis*, which produced short chains and was generally non-pathogenic. This scheme was extended by other workers, who introduced such terms as *Strept. longissimus* and *Strept. brevissimus.* This method of classification is no longer tenable. The morphological arrangement of the various species is not constant ; in fact, a single strain may exhibit all the so-called " characteristic arrangements " when grown under different cultural conditions.

Another method of classification was based on the power of the different species to ferment the various carbohydrates. Gordon (1903), Andrewes and Horder, and later Holman (1916) introduced different schemes, but none has proved satisfactory as the sole basis of classification. The results obtained by different workers have tended to be irregular, and it seems to be doubtful if the fermentative properties of a given strain are absolutely permanent. Fermentation tests are sometimes used as a basis for sub-grouping freshly isolated strains.

Schottmüller (1903) differentiated the streptococci by their capacity to produce hæmolysis when grown on blood-agar plates. Two types were described: (1) *Strept. hæmolyticus* or *longus*, which produced definite lysis of the R.B.C. and was usually arranged in long chains, and (2) *Strept. viridans* or *mitior*, which gave colonies surrounded by a greenish zone and tended to appear in short chains. This scheme was elaborated some time later by Smith and Brown (1919). They found that poured plates of blood-agar containing deep colonies were superior to surface growths. Under such circumstances three distinct appearances were observed :—

(1) "*Alpha*" type, in which the individual colonies were immediately surrounded by a zone of green discoloration, on the outside of which was a thin, clear zone of complete lysis. The outside clear zone sometimes failed to develop in 24 or 48 hours. This corresponded to the *Strept. viridans* of Schottmüller.

(2) "*Beta*" type, around the colonies of which was a definite clear zone of complete hæmolysis. This was identical with the *Strept. hæmolyticus* of Schottmüller.

(3) "*Gamma*" type, in which no change occurred in the medium surrounding the colonies. A number of these organisms is normally present in the intestinal tract of man, and they have been grouped together as *Strept. fæcalis* or *enterococcus*.

The green discoloration in "α" hæmolysis on unheated blood-agar plates has not been satisfactorily explained, but it probably results from the formation of a green pigment by met-hæmoglobin. With heated blood-agar the greenish zone is caused by the action of hydrogen peroxide produced during the growth of the organism, as the catalase of the blood is destroyed by the heating process in the preparation of the medium. Some α strains may produce zones of complete lysis on surface growth, but they do not, however, form a soluble hæmolysin; these are sometimes referred to as *pseudo-hæmolytic* strains. It has consequently been found that the action of fluid cultures on a saline suspension of corpuscles is a better test of hæmolysis than the action on a blood-agar plate. Lysis is only produced by strains giving the β type of hæmolysis by the plate method. Some workers have consequently divided the streptococci into two main groups—(1) hæmolytic and (2) non-hæmolytic, which includes the "α" or *viridans* strains and the "γ" or *non-hæmolytic* strains.

The changes produced in blood-agar tend to be reasonably constant, and this criterion has consequently been almost universally adopted as a basis for the primary sub-division of the *Streptococci*. It is, however, important to note that the action on blood corpuscles has no real fundamental significance; it appears to be possible on certain occasions to render some hæmolytic streptococci non-hæmolytic by inoculation into the tissues.

Much evidence is now accumulating to indicate that each subgroup, especially *Strept. hæmolyticus*, contains a number of different types. Fermentation reactions and serological tests such as agglutination, precipitation and complement-fixation, have been employed repeatedly in efforts to sub-divide the hæmolytic streptococci. While many discrepancies were present in the results of the early serological reactions, recent investigations have been more satisfactory. Two factors are mainly responsible for the frequent irregularities in the tests; in the first place stable suspensions are difficult to prepare and spontaneous agglutination is a common occurrence; secondly, it has recently been found that the streptococci possess a very complex antigenic structure. Lancefield has identified three main fractions in human strains—(1) a type-specific protein "M," (2) a non-specific nucleo-protein "P," and (3) a group-specific carbohydrate "C"; while Mudd and his colleagues have obtained another distinct fraction which is labile and phagocytosis-promoting. Nevertheless, thanks largely to the efforts of Griffith and Lancefield, the sub-grouping of the hæmolytic strains by serological methods is now being firmly established.

The terminology of the genus *Streptococcus* has been rendered chaotic by the adoption of such terms as *Strept. scarlatinæ*, *Strept. erysipelatis*, etc. There is, however, no justification for this practice, and it is to be hoped that the use of these terms will soon be abandoned, as it is impossible to separate the streptococci on clinical grounds. One term, *Strept. pyogenes*, has been adopted on the grounds of priority by many workers to represent the pathogenic human hæmolytic streptococci; the use of this term is only tentative, but it serves a useful function in distinguishing the human strains from those of animal origin. It corresponds with the group A of the Lancefield classification.

Morphology. *Streptococci* are spherical cocci, 0·6–1μ being the average diameter, and tend to be arranged in chains the length of which is subject to considerable variation. *Strept. fæcalis* strains usually occur in pairs or short chains consisting of three or four cocci, while the various strains of *Strept. hæmolyticus* and particularly *Strept. viridans* may exhibit marked irregularities. Some occur in chains containing twenty or thirty organisms, while others are found in very short chains. *Streptococci* are non-motile, non-sporing, and do not usually exhibit capsule-formation. They are readily stained by the simple dyes and retain the Gram stain, *i.e.*, they are Gram-positive.

Many strains of *Strept. hæmolyticus* form a capsule, the main constituent of which is hyaluronic acid, and produce mucoid colonies.

Cultural Characteristics. Many species of *Streptococci*, particularly the pathogenic members, do not grow well on the ordinary media. Enrichment of the simpler media with serum, ascitic fluid or blood is therefore necessary. Some relatively non-pathogenic varieties, such as *Strept. fæcalis*, grow luxuriantly on the simple media. Multiplication as a rule takes place over a moderate range of temperature, 20°–42° C., but great variation is exhibited by the different strains. The optimum temperature in most cases is 37° C. The majority of members are facultative anaerobes and grow best in the presence of free oxygen. *Anaerobic* strains exist and have been cultivated from cases of puerperal fever and lung abscess. These strains have received little attention and the various types cannot be satisfactorily differentiated. The optimum reaction of the medium for growth is pH 7·4.

In *fluid media* the character of the growth varies with the particular species. The *hæmolytic* strains tend to give a granular deposit with a clear supernatant fluid. The *non-hæmolytic* strains frequently show a well-marked general turbidity with only a slight deposit. *Viridans* strains often present both features, *i.e.*, a deposit together with a slight general turbidity. In almost all instances there is a definite relationship between the cultural appearance in a fluid medium and the length of chain-formation; with long chains a deposit tends to form, while short chains are usually associated with a general turbidity.

On *solid media* the *hæmolytic* and *viridans* strains as a rule form small (0·5–0·75 mm.), opaque, raised, circular colonies with a granular structure; growth is never profuse. *Strept. fæcalis* strains tend to grow more prolifically, the colonies are larger, more opaque and smoother in appearance. On blood-agar not only do the different species produce their respective changes in the medium immediately surrounding the colonies, but, in the case of the *hæmolytic* strains, there may be characteristic changes in the colonial appearance. This applies particularly to the strains pathogenic for man. Todd found that recently isolated organisms gave a granular, irregular, roughish type of colony, to which he gave the name "matt". After sub-culture the virulence was sometimes lost without any alteration occurring in the colonial appearance; these he termed "matt-attenuated" variants. In other cases the loss of virulence was associated with changes in the form of the colonies, which became smooth, regular and glossy; these Todd termed "glossy" variants. The change from the "matt" to the "glossy" form was later found to be associated with the loss of one of the antigenic components ("M" type-specific factor) of the organism.

Biochemical Activities. *Streptococci* as a whole do not exhibit marked metabolic activities. They ferment many carbohydrates with the production of acid only, but they have few proteolytic properties and pigment formation is not found. Indole is not produced, and most members do not liquefy gelatin; the power to liquefy gelatin is, however, possessed by some *fæcalis* strains. Some strains of *Strept. fæcalis* also produce H_2S.

Fermentation reactions have been used as a basis of sub-division by many workers. The sugars particularly useful in this respect are lactose, mannite, salicin and inulin. Salicin is fermented by most *hæmolytic* strains and mannite by *fæcalis* strains; lactose is not broken down by equine strains of *Strept. hæmolyticus*, while inulin is sometimes useful in differentiating the *pneumococci* from the green-producing streptococci. Litmus-milk is acidified by most members, but is clotted only by *fæcalis* strains. Recent work suggests that *hæmolytic* types of human and bovine origin may be differentiated by their action on sorbitol and trehalose. Most, but not all, bovine strains, which are non-pathogenic for man, ferment sorbitol but not trehalose; human varieties ferment trehalose and not sorbitol.

Resistance. Most streptococci are very susceptible to destructive agents; heat at 55° C. for 15–20 minutes being sufficient to kill them. *Strept. fæcalis*, however, offers a relatively high resistance for a non-sporing organism; it can withstand a temperature of 60° C. acting for 30 minutes.

In the conservation of stock-cultures little difficulty is experienced with *Strept. fæcalis*, which can be kept at room-temperature for many weeks. *Hæmolytic* and particularly *viridans* varieties, however, require careful attention and frequent sub-culturing when kept on serum- or blood-agar. They may be conserved without difficulty either by growing in Robertson's meat medium and subsequently placing in the refrigerator or by drying *in vacuo*.

Serology. In spite of the enormous amount of work carried out, the serological investigations of the streptococci have until recently been disappointing. Agglutination has been complicated by the difficulty in obtaining stable bacterial suspensions, but this has been overcome by different workers with varying degrees of success. Griffith employed the slide method of agglutination with cultures prepared daily in broth, serum-broth and ascitic fluid-broth. In this way spontaneous agglutination was overcome and he was able to classify the *hæmolytic* strains causing disease in man ; some 30 significant types were described and practically all belong to Group A. Irregularities were ascribed chiefly to variations in type specificity. It is important to note that the performance of these tests and the interpretation of the results are not simple, and that considerable experience is necessary in order to obtain consistent and reliable readings. Typing is particularly valuable in epidemiological surveys.

Precipitation tests have recently been gaining favour, due largely to the observations of Lancefield. She demonstrated that the antigenic structure of the hæmolytic streptococcus was extremely complex, three well-defined immunological components being isolated :—

(A) A type-specific protein " M " which, in the case of " matt " group A strains, differentiates types in agreement with the agglutination tests.

(B) A non-specific nucleo-protein " P," which reacts with similar fractions from other Gram-positive cocci, such as *Strept. viridans*, *Staphylococcus* and the pneumococcus.

(C) A group-specific carbohydrate " C."

Using precipitation tests in which the group-specific " C " substance was the antigen, Lancefield was able to distinguish five groups of *hæmolytic streptococci*, viz. A, B, C, D and E. Group A contained almost entirely strains from human sources. These results were confirmed later by Lancefield and Hare, who also described further new groups F, G, H and K, which were isolated from human sources, but apparently had little pathogenic activity.

These studies are continuing and some 12 groups have now been identified. It is, however, generally accepted that Group A strains are the important human pathogens. Group B strains are often found in raw milk, as they are responsible for bovine mastitis; Group C strains are obtained from the disease in horses known as strangles.

Toxin Production. Various toxic substances are produced by the *hæmolytic streptococci*, particularly group A strains, viz., hæmolysin, leucocidin, fibrinolysin and the erythrogenic toxin. Some strains also produce the enzyme, hyaluronidase, but this does not appear to be connected with the virulence of the organisms; such strains are non-capsulated.

The *hæmolysin* is formed during the early stages of growth; in a suitable medium the maximum formation is reached in about 10–14 hours. The hæmolysin is relatively labile, being destroyed by heat at 55° C. Its pathogenic function is uncertain; it has been suggested by some workers that the hæmolysin is responsible for the pyogenic action, and by others that the hæmolysin is related to virulence. The validity of these observations is uncertain; it is, however, interesting to note that the actively pathogenic streptococci are hæmolytic, but that not all hæmolytic streptococci are pathogenic. It has recently been found that hæmolytic streptococci may form two distinct types of hæmolysin, one being oxygen-sensitive and the other acid-sensitive.

The *leucocidin* is also produced during the early stages of growth, the maximum concentration in serum-broth is reached in 10–18 hours. It is inactivated by heat at 60° C. in 30 minutes. The polymorphonuclear leucocytes are susceptible, but the macrophages are resistant, to its action.

The *fibrinolysin* of pathogenic strains can liquefy clotted normal human plasma in 10–45 minutes. The serum of patients recently recovered from acute streptococcal infections is able to inhibit this fibrinolysis, while the plasma of these cases is resistant to the fibrinolytic action.

The *erythrogenic toxin*. It has now been definitely established that certain strains of hæmolytic streptococci produce an active toxin, which resembles in many respects the so-called "exotoxins". Early observations indicated that some of the hæmolytic streptococci might produce a toxin under certain conditions, but the results were vague and indefinite. When the Dicks established that a hæmolytic streptococcus was the ætiological agent of scarlet fever interest was again renewed. As a result it was found that strains, isolated not only from cases of scarlet fever but also from other sources, produced a toxin to which the terms "Dick," "scarlatinal," "streptococcal" and "rash-producing or erythrogenic" toxin have been applied. Of these the term suggested by Okell, "erythrogenic" toxin, appears to be the most appropriate.

The erythrogenic toxin is filterable and antigenic, but it differs from most exotoxins in that it is relatively heat-stable. It resists heating at 75° C. for 10 minutes, and is only inactivated by heating at 96° C. for 45 minutes. Investigations of the properties of this toxin have been greatly hampered by the lack of satisfactory laboratory animals, none of which appears to be susceptible to intradermal injections; goats and white pigs have been employed by some workers, but the results have proved irregular. The toxin has a lethal effect on rabbits following intravenous inoculation, but the doses required are relatively high, 5 c.c. or more. Man, however, is particularly susceptible, and in many laboratories susceptible volunteers are employed for the standardization of the toxin and antitoxin; on intradermal injection of the toxin, suitably diluted, a local erythematous patch, appearing in a few hours and reaching a maximum within 24 hours, is produced. In America a *skin-test-dose* (S.T.D. = unit of toxin) is taken to be the amount of toxin necessary to produce such a reaction 1 cm. in diameter about 24 hours after intracutaneous injection of individuals susceptible to scarlet fever. A unit of antitoxin is ten times the smallest amount of antitoxin required to neutralize a "*test dose*," which is five skin test doses of toxin; thus 1 antitoxin unit = 50 skin test doses of toxin. In Great Britain there is as yet no official standard.

In America and several other countries the preparation of the toxin and antitoxin is controlled by the patents obtained by the Dicks, who adopt their own independent standards in testing these products. Under such circumstances it is impossible to fix an international standard and unit.

The intradermal injection of the toxin is now widely employed to detect individuals susceptible to scarlet fever; this is termed the "Dick test". Small doses of the toxin produce a local reaction, but when large doses are inoculated into susceptibles a miniature attack of scarlet fever may be produced.

The toxin can be readily prepared by growing a toxigenic strain for 3–5 days in broth (pH 7·5) containing 5 per cent. defibrinated rabbit's blood. It can be concentrated and purified, and has apparently no relation to the hæmolysin, nor has it any pyogenic properties. There has been much controversy as to the unity or diversity of the toxins produced by the different strains of hæmolytic streptococci, and the question is still unsettled. Much evidence is, however, available to indicate that only one erythrogenic toxin is produced, but that the amount formed by the various strains differs considerably; *i.e.*, the difference is quantitative rather than qualitative. As one might expect, strains isolated from cases of scarlet fever usually produce the toxin more abundantly than strains isolated from other sources.

Pathogenicity. The different species of streptococci vary considerably in their pathogenicity for man and animals.

Strept. non-hæmolyticus strains are present in the upper respiratory tract and the intestines. They are commensals and generally have a low degree of pathogenicity for man. They have been isolated occasionally in blood-cultures from cases of rheumatism, but their significance is uncertain. *Strept. fæcalis* strains are members of normal intestinal flora, they are relatively non-pathogenic and, when associated with a disease process, occur mainly as secondary invaders. They are, however, not infrequently responsible for the development of cystitis and perhaps pyelitis.

The various strains of the *Strept. viridans* are usually present in the upper respiratory tract and are not very active pathogens; some, however, are often found in man in association with subacute bacterial endocarditis, apical dental abscesses, and also as secondary invaders in infections of the respiratory tract. They have been incriminated as the ætiological agents of many diseases of unsettled origin, such as gall-stones, gastric ulcers and various forms of rheumatism. Conclusive evidence of such associations has, however, never been produced. It has been found that *Strept. viridans* can frequently be isolated by blood-culture a short time after tooth-extraction. This is considered to be an important factor in the development of malignant endocarditis; there is much evidence that the organisms, entering the blood-stream following the extraction of teeth, gain access to pre-existing cardiac vegetations.

Strept. hæmolyticus is an extremely important pathogen of man and animals, in which it is responsible for many common and serious conditions. The manifestations of infection are, however, extremely varied. In man, on gaining entrance into the subcutaneous tissues, *Strept. hæmolyticus* may cause a spreading form of cellulitis in which generalization of the infection and septicæmia are not uncommon. Strains of *Strept. hæmolyticus* are also responsible for such diverse conditions as scarlet fever, puerperal fever, erysipelas, sore throat, tonsillitis, meningitis, pneumonia, empyema and otitis media; these strains almost invariably belong to Group A.

Thanks largely to experiments on human volunteers by the Dicks, it is now generally accepted that in scarlet fever the infection remains localized in the throat, while the general symptoms are mainly due to the production of the erythrogenic toxin, which is absorbed into the blood-stream and gives rise to the characteristic rash from which the name of the disease has been derived. The differences in the clinical manifestations of streptococcal sore throat and scarlet fever are due largely to variations in the toxigenicity of the infecting strains and the resistance of the host.

Strept. hæmolyticus may also cause such serious secondary infections as broncho-pneumonia following measles, whooping-cough and influenza. Some workers have suggested that the hæmolytic streptococcus is concerned with the ætiology of acute rheumatic fever (p. 422).

Our present conception of human streptococcal infections is based on the unitarian hypothesis, which postulates that the various diseases may be caused by any strain of *Strept. hæmolyticus* (Group A). The old view that certain streptococci had a special disease specificity has long been discredited and such terms as *Strept. erysipelatosus*, *scarlatinæ* and *puerperalis* are obsolete. The different clinical manifestations are due, on the one hand, to variations in one or more of the offensive weapons of the organism, and, on the other, to changes in the susceptibility of the infected individual. To illustrate this hypothesis it is considered that in scarlet fever the erythrogenic toxin, in empyema pyogenicity, and in septicæmia invasiveness are respectively the predominant pathogenic factors. The unitarian hypothesis is strongly supported by serological observations. Disease-specificity by different strains has not been found; serologically identical strains have been isolated from cases of scarlet fever, sore throat, empyema, puerperal fever and otitis media. Ward outbreaks have been reported in which, following the introduction of cases of acute streptococcal infections, such conditions as septic pharyngitis, erysipelas, scarlet fever and otitis media have developed in other occupants of the wards; in all cases the type of the invading streptococcus has been the same as that of the original case.

It has been noted that the severity of many streptococcal infections, particularly scarlet fever, has decreased considerably during the past half-century. The probable explanation of this is that, as streptococcal infections are common in crowded communities, a resultant increase in the resistance of the community, particularly to the erythrogenic toxin, has developed.

Diagnosis. In routine practice the identification of the various species of streptococci is in most cases incomplete. While the primary sub-division into hæmolytic, viridans or non-hæmolytic strains is carried out, the type, in the case of the hæmolytic varieties, is not usually determined.

In the case of pus and swabs, the examination of smears and the preparation of cultures on blood-agar plates are the usual methods of investigation. The appearance around any colony of complete or partial hæmolysis is noted and sub-cultures of these on to blood-agar should be made. In order to verify the type of hæmolysis it is advisable to test a broth culture against a saline suspension of the corpuscles. As previously mentioned, the pseudo-hæmolytic strains of *Strept. viridans* may give zones of complete lysis on blood-plates and so appear as hæmolytic strains. In view of the recent observations on the *rôle* of *anaerobic streptococci* in the ætiology of puerperal fever, cultures should be incubated under both aerobic and anaerobic conditions.

In making blood-cultures, in such conditions as malignant endocarditis or septicæmia, the blood is collected under aseptic conditions from the median vein, and 5 c.c. are placed into a flask of broth. For a quantitative examination 1 c.c. of the blood is added to a small amount of glucose broth and trypsin, which prevents coagulation ; this is then mixed in a Petri dish with melted agar, and after incubation at 37° C. for 24–48 hours the colonies are counted.

The identification of *Strept. hæmolyticus* is a relatively simple question, but, as all hæmolytic strains are not pathogenic to man, it is important that a means of differentiating pathogenic from non-pathogenic strains should be available. This can be readily appreciated when it is realized that hæmolytic streptococci are normally present in the naso-pharynx of approximately 20 per cent. of normal individuals, but approximately one-third of these strains only are potential pathogens and fall into the group A of Lancefield. Methods used for this purpose, particularly in the case of strains isolated from throat swabs, are :—

(1) The precipitin tests of Lancefield, using the " C " precipiti-

nogen, to determine whether the strain isolated belongs to group A.

(2) The agglutination method of Griffith, by which group A strains are typed.

(3) The biochemical and fibrinolysin tests used by Hare and Colebrook; these include the fermentation of sorbitol and trehalose, growth on 40 per cent. bile-blood-agar, and the digestion of human fibrin, and serve to differentiate human from bovine strains.

The precipitin test is particularly valuable for determining whether hæmolytic streptococci isolated from the throats of healthy individuals are potential pathogens. The agglutination test is useful for tracing the source and extent of an outbreak.

To obtain extracts containing the group factor, *i.e.*, the carbohydrate "C" of Lancefield, seed the organisms in 20 per cent. serum trypsin broth and incubate at 37° C. for 18 hours. Centrifuge and to the deposit add 1 c.c. of N/20 HCl. and heat in a water-bath at 100° C. for 10 minutes; cool and centrifuge. Take the supernatant fluid and neutralize with N/1 NaOH. Precipitation can be readily demonstrated by the capillary pipette method.

Schultz-Charlton Reaction. The diagnosis of scarlet fever may be assisted by the application of a specific skin test. Before the ætiological relationship between the hæmolytic streptococcus and this disease was definitely established, Schultz and Charlton found that the intradermal inoculation of serum, collected from convalescent cases, into a patient showing the erythematous rash resulted in local blanching. This phenomenon was termed the "Schultz-Charlton reaction", and was later found to be given also by streptococcal antitoxin. The antitoxin has been used in practice as a diagnostic measure, but, as it has been shown that blanching only occurs consistently when the rash is typical, this test has a limited practical value.

Prophylaxis. In recent years many prophylactic measures have been adopted to prevent streptococcal infections. As one of the general measures, the propaganda against post-mortem wounds may be cited; slight wounds received in the post-mortem room have resulted in streptococcal infections in which invasiveness has been a characteristic feature. The number of these infections has been reduced by drawing the attention of workers to the potential danger of these wounds.

In the case of *puerperal fever* it is now considered that infection takes place mainly from the naso-pharyngeal droplets of the attendants at the confinement or from the naso-pharynx of the patient herself. The human upper respiratory tract appears to be the main habitat of the group A strains. In view of these observations, it is now the practice to swab the throats of all attendants of maternity homes or wards and to exclude carriers of hæmolytic streptococci. The possible fallacies of this procedure, as at present generally applied, and the importance of group A strains have already been discussed, but, even as carried out at present, this practice has doubtless had definitely beneficial effects. It is important to note that, if a case of puerperal fever is reported in a ward, the swabbing of the staff is essential and all reported carriers must be isolated. Masks should be worn during any operative procedure, including the dressing of wounds.

In the case of *scarlet fever* carriers of hæmolytic streptococci are detected, but the significance of the results is uncertain until the laboratory tests are more informative. Griffith's types I, II, III, IV and V are usually responsible for scarlet fever in this country. In susceptible communities, such as schools, it has been shown that in any one term a school tends to have one main epidemic type of streptococcus. It has also been found that, in many cases, complications of scarlet fever are caused by cross-infection with strains other than the original streptococcus. Because of this home-nursing of cases of scarlet fever is becoming more widely adopted.

A valuable step in the prophylaxis of scarlet fever has been the introduction of the "*Dick test.*" This consists in the intradermal injection of one skin-test dose of a potent erythrogenic toxin, diluted with saline and contained in 0·2 c.c. As a control the same dose of the toxin heated at 100° C. for 1 hour may be used. The appearance of an erythematous patch (1–2 cm. in diameter) on the test area in 18–24 hours and absence on the control is a positive reaction and indicates susceptibility to scarlet fever ; a negative reaction is indicative of resistance. Pseudo-reactions are extremely uncommon as, in view of the dilution of the toxin, little extraneous protein is present in the inoculum. Having determined the susceptibles, the question of immunization arises. This applies mainly to scarlet fever, which is essentially a toxæmia ; immunization may consequently be carried out either passively by the use of antitoxic serum or actively by means of the toxin.

Active immunization is performed by the subcutaneous injection of increasing doses of the toxin. This practice has not received official recognition, and no generally accepted scheme is available. The main reasons for this are that a satisfactory toxoid is rather difficult to prepare owing to the marked resistance of the toxin to the action of formalin, and, also, as previously stated, a satisfactory method of standardization is not available.

Active immunization has, however, been tried extensively on the nursing staffs of fever hospitals, and the results are considered by many to be satisfactory. Large doses are essential; the Dicks recommended an initial dose of 500 skin test doses, increasing to 80,000–100,000 skin test doses in the course of five injections. Under such treatment it is stated that 95 per cent. of the susceptibles become Dick negative within 2 weeks after the final injection and usually remain immune for from 1 to 5 years.

Passive immunization by the injection of streptococcal antitoxin has been used mainly to prevent the spread of scarlet fever in hospitals. Contacts are Dick tested, and all susceptibles are given 5–10 c.c. of the serum subcutaneously. The duration of immunity is, however, very short, 7–14 days being the usual time. It has been found that as the serum does not influence the infecting organism, attacks may develop when the temporarily increased resistance passes. Passive immunization has consequently not been very popular.

Specific Therapy. Antitoxic and antibacterial sera have been repeatedly tried in the therapy of streptococcal infections, but usually with unsatisfactory results. *Antitoxic sera* have been more valuable than the antibacterial types and are particularly indicated in the therapy of infections, such as scarlet fever, in which the erythrogenic toxin is mainly responsible for the symptoms. Scarlet fever has in recent years been a relatively mild condition, and striking demonstrations of the efficacy of serum-therapy have therefore been difficult to obtain. There is therefore some diversity of opinion as to its value as a means of saving life. Many workers have found that the incidence of complications has been reduced by the early administration of the antitoxic serum, which, however, has no effect on complications already established. It will be impossible to form a final opinion of the value of antitoxin therapy in scarlet fever until a severe epidemic, having a high mortality rate, appears.

Polyvalent antibacterial sera prepared in horses have been used for many years in the therapy of acute streptococcal infections, in which invasiveness is a predominant feature. The results, however, have been extremely disappointing. This is not unexpected, as it is now recognized that the hæmolytic streptococcus presents a great variety of antigenic types and, to be effective, an antibacterial serum must at least possess the homologous type-specific antibody of the infecting organism. Some of these acute cases have improved after the inoculation of the antitoxic serum, but this type of serum is frequently without effect in infections other than scarlet fever.

Chemotherapy. Infections with *Strept. pyogenes* respond well to chemotherapeutic agents. The sulphonamides give excellent results ; oral administration is generally satisfactory, but the intravenous route may be necessary in fulminating infections.

Strept. pyogenes is sensitive to penicillin which should be given when the response to sulphonamides is poor, as in meningitis or infections with sulphonamide-resistant strains. Penicillin is also proving valuable in subacute bacterial endocarditis caused by *Strept. viridans :* high and prolonged dosage, such as 14 megaunits in a month, is necessary.

CHAPTER XVI

PNEUMOCOCCUS : PNEUMONIA

Pneumococci are Gram-positive cocci, exhibiting a marked tendency to arrangement as lanceolate diplococci and growing poorly on ordinary media ; they usually ferment inulin, are soluble in bile and are frequent pathogens in man.

Classification. Pasteur (1881) during his work on rabies described a capsulated diplococcus, which he found in the saliva of a child. Fraenkel (1886) saw a similar organism in the sputum of a pneumonia patient ; but the association of the organism with pneumonia was definitely established by Weichselbaum (1886), who also found that it was normally present in the mouth. To this organism he gave the name *Diplococcus pneumoniæ.* Schottmüller (1903) described an organism which he termed *Strept. mucosus ;* this is now accepted as a pneumococcus (type III). These observations were readily confirmed, and the presence of the pneumococcus in the sputum of normal individuals and also its *rôle* as the ætiological agent of pneumonia became definitely established. Our knowledge was further extended when some American workers, in 1917, found that the pneumococcus could be sub-divided serologically into a number of distinct types, viz. types I, II, III, plus a heterogeneous collection referred to as group IV.

The taxonomic position of the pneumococcus is somewhat uncertain. In the classification issued by the Society of American Bacteriologists it is included as the type species of the genus *Diplococcus.* This arrangement is, however, not universally accepted ; many workers consider that it is a species of the genus " Streptococcus ", as it bears a close resemblance in some respects to the *Strept. viridans.* There are, however, several well-marked differences, *e.g.*, bile-solubility, inulin-fermentation and agglutination, which are reasonably constant and appear to place the pneumococcus in a separate genus from the streptococci.

Morphology and Staining Reactions. When freshly isolated from the tissues the pneumococcus is usually arranged in pairs, each coccus being more oval or lanceolate than spherical, with the broader ends opposed; each pair is usually enclosed in a single capsule. On prolonged cultivation on artificial media, particularly the fluid varieties, the cocci tend to become more spherical and to be arranged in short chains of three to five.

The pneumococcus is non-motile, having no flagella, and does not form spores. Capsule-formation is usually well marked on fresh isolation from the tissues, particularly with type III strains; the power to form capsules, however, becomes considerably decreased on prolonged artificial cultivation. The pneumococcus stains readily with the usual dyes and it is strongly Gram-positive. The capsules are not stained by the ordinary staining methods, and, when present, are represented by unstained haloes around each pair of cocci; they can, however, be readily demonstrated by special methods, such as those of Muir and Hiss (*q.v.*).

Cultural Characteristics. The pneumococcus can usually be cultivated without much difficulty. It is important to note that, on first isolation, growth on the simpler media is frequently very poor. Enriched media, such as serum-agar or blood-agar, are therefore employed. Growth may be obtained throughout a temperature range of 25°–40° C., but the optimum temperature is 37° C. The optimum reaction of the medium is *p*H 7·6–7·8. The organism is a facultative anaerobe, and consequently maximum growth takes place in the presence of free oxygen.

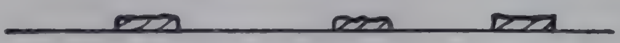


FIG. 14.—Colonial appearance of the pneumococcus (in section).

In *fluid media*, such as Hartley's broth or serum-broth, growth in 24 hours usually occurs as a well-marked general turbidity. On further incubation the medium may become clear, owing to autolysis of the organisms.

On *solid media* growth is frequently delicate; the colonies are small, round and semi-transparent. On blood-agar, when freshly isolated, a more or less typical appearance is found; the colonies are flat with a sharply raised edge, which tends to be raised above the rest of the colony. On further incubation a central raised portion frequently becomes differentiated from the remainder of the colony (Fig. 14); also around the colonies the α type of hæmolysis is found.

The colonies of the pneumococcus (type III) are generally mucoid, owing to the large amount of capsular substance formed by these strains. In all cases old cultures may give atypical appearances.

Biochemical Activity. The pneumococcus exhibits little demonstrable proteolytic activity ; gelatin is not liquefied and indole is not formed. Its fermentation activities are more marked ; lactose, maltose, glucose, sucrose and inulin are fermented by most strains with the production of acid, but not gas. As growth is difficult to obtain in the usual peptone-water-sugars an enriched medium is required. The one in frequent use is Hiss's serum-water, which contains 1 part of serum to 3 parts of water, to this the sugar and indicator are added ; acid formation is indicated by a change in the colour of the indicator and coagulation of the medium. Litmus milk is acidified and frequently clotted.

Resistance. The pneumococcus is readily destroyed by bactericidal agents, being killed by a temperature of 56° C. in 15–20 minutes. It is a delicate organism and the conservation of stock-cultures is not easy. A satisfactory method of preservation in stock-culture is the inoculation of a medium composed of semi-solid agar to which fresh rabbit's blood has been added, and conservation, without incubation, in the refrigerator. Under such conditions survival may last for several months. Drying *in vacuo* at a low temperature gives excellent results.

Bile-solubility. It has long been recognized that the addition of bile salts to a culture of the pneumococcus in a fluid medium results in lysis of the organisms. This property is now used extensively as a means of identification. The mechanism of the action has not been definitely determined, but it is thought that the bile salts act by accelerating the normal autolytic process of the bacterial cell. The lytic activity of bile salts appears to be related to their hydroxyl grouping. Salts having no hydroxyl groups are inactive ; salts with hydroxyl groups, such as desoxycholic acid and apocholic acid, are very active. Bile-solubility is inhibited by heating the culture for 30 minutes at 56° C.

In carrying out the test, a small amount (2–4 drops) of ox-bile or a suitable bile salt, diluted 1/10 or 1/100, is added to 5 c.c. of a broth culture of the pneumococcus, which, after standing about 10–15 minutes at 37° C., should be cleared. The reaction of the culture should not be below *p*H 6·6, as, at this point, precipitation of the bile salts takes place.

Serology. The existence of different serological strains was first noted by Neufeld and Handel (1909). These preliminary observations were elaborated by Dochez, Avery and others in

America, and Lister in South Africa. The American workers (1913 onwards) found that there were three common infecting types of pneumococcus, which they labelled I, II and III; in addition, a heterogeneous group, labelled group IV, was described. Lister (1916), investigating pneumonia in South Africa, described three main serological types, which he styled A, B and C. It was subsequently demonstrated that type B corresponded with the American type II, and type C with type I, but type A had apparently no American counterpart. These observations were soon confirmed, and their importance in the epidemiology of pneumonia recognized. The American system of classification was subsequently adopted, and it is now widely employed. Some twenty-nine types have been identified in group IV, *i.e.*, there are in all thirty-two distinct types; in two instances it appears that strains, given a separate identity, correspond with strains previously described and therefore only thirty distinct strains have been isolated.

Investigating the antigenic structure of the various types of pneumococcus, Heidelberger, Avery and their co-workers (1923) made a striking discovery. They found that the pneumococcus contains two main antigenic components: (1) a nucleo-protein and (2) a complex carbohydrate; other factors are undoubtedly present, but their significance is uncertain. It was also found that the protein is common to all types, but the carbohydrate is distinct and specific to each. The carbohydrate has the complex chemical structure of a polysaccharide and has been termed "specific soluble substance" or "S.S.S." The carbohydrate in the case of the type I pneumococcus is dextro-rotatory and contains nitrogen; that of type II is dextro-rotatory, nitrogen-free, and on hydrolysis yields a glucose unit; that of type III is lævo-rotatory, nitrogen-free, and on hydrolysis yields glucose and aldobionic acid. Moreover, this substance was found to be intimately associated with the capsule of the organism and to behave as a *haptene*, *i.e.*, it is a partial antigen, reacting *in vitro* with the specific antibodies produced by the homologous organisms, but unable alone to stimulate their production *in vivo*. The smooth to rough (S → R) variation is accompanied by a loss of the capsule and virulence. The type-specificity is thus lost and, as a result of the presence of the common nucleo-protein fraction, a reaction is obtained with antisera prepared against autolysed cultures of any pneumococcal type.

The serological tests used extensively in the study of the pneumococcus are agglutination and precipitation; in the former test the whole bacterial cell is involved, and in the latter the specific soluble substance.

Toxin Production. The pneumococcus does not produce a soluble toxin. A substance similar in many ways to an endotoxin has, however, been extracted from the organism by autolytic dissolution or by alternate freezing and thawing. It has been suggested that the toxic symptoms, which are so frequently present in pneumococcal infections, may be due to absorption and diffusion of the specific soluble substance. The "S.S.S." has been found to be closely associated with the virulence of the organism, and it has been detected in the blood-stream and the urine of pneumonia patients, but proof that it is responsible entirely for the toxic symptoms has not been obtained (*vide infra*).

The pneumococcus produces a soluble hæmolysin. It can be demonstrated in young broth cultures, but as it is readily inactivated by oxidation it is soon destroyed in aerobic cultures. On blood-agar its action is naturally most marked when grown under anaerobic conditions. The hæmolysin is not type-specific and appears to be independent of virulence. Its exact function, particularly in the pathogenesis of pneumococcal infections, is obscure. It is antigenic and an anti-hæmolysin has been demonstrated in the blood of pneumonia patients.

Pathogenicity. In man the pneumococcus is normally found as a commensal, being present in the mouth and naso-pharnyx of many healthy individuals. It is also responsible for many diverse conditions such as lobar pneumonia, broncho-pneumonia, empyema, otitis media, meningitis, arthritis, endocarditis and primary peritonitis of children.

The pathogenicity of the pneumococcus for laboratory animals varies considerably. Rats are highly resistant, while white mice and rabbits are extremely susceptible. In white mice intraperitoneal injection of a fresh virulent strain is followed by a purulent peritonitis, septicæmia and death in 24–48 hours. The normal resistance of man appears to be midway between these extremes.

Pneumonia

Lobar pneumonia, in approximately 90–95 per cent. of cases, is caused by the pneumococcus. The pneumococcus is also found as a commensal in the mouth of some 20–50 per cent. of normal individuals, but it is interesting to note that the incidence of the various types of the organism in pneumonia is in marked contrast with the incidence of the types in the nasopharynx of normal individuals. (Table VI.)

It is seen that some 60–75 per cent. of pneumonia cases have been caused by the classical types I, II and III. The figures have varied in the different countries, particularly in the case of type III infections which have been uncommon in this country but relatively frequent in U.S.A. A study of cases occurring in U.S.A. during recent years has shown that the type I pneumococcus was consistently the most prevalent; other common types were II, III, V, VII and VIII.

TABLE VI

Frequency of the Various Types of the Pneumococcus in Lobar Pneumonia

Country	Years	No. of Cases	Percentage incidence of types			
			I	II	III	Group IV
Glasgow (Cruickshank) . .	1930–32	1,215	36·2	35·5	3·6	24·7
Salford (Langley, Mackay & Stent)	1932–37	800	50	25	1	24
U.S.A. (Finland) .	1929–36	2,194	30	13	15	42

The type incidence of pneumococci in normal individuals is quite different; the majority of strains belong to Group IV and, while type III is relatively common, types I and II are rarely found. Extensive surveys in different countries have shown that, although the carrier-rate in a normal population may vary over a wide range, roughly 20–60 per cent. are the usual figures, the distribution of types has shown a similar trend. It has also been found that an individual may harbour two or more distinct types at the same time, and that strains may either persist in the nasopharynx for long periods or disappear rapidly.

The relative frequency of types I and II in pneumonia and their rarity in the mouths of normal individuals indicate that infection in pneumonia is not endogenous, but is obtained from an outside source, *i.e.*, it is exogenous. The organism is generally conveyed from healthy or convalescent carriers by droplet infection. Resistance is normally high, and only when this has been reduced by such non-specific causes as fatigue, alcoholism and under-nourishment does pneumonia usually develop. Recent observations indicate that pneumonia is rarely a primary disease but is usually preceded by some infection of the upper respiratory tract, such as the common cold, laryngitis or influenza. These infections are considered to facilitate the spread of the pneumococcus from the naso-pharynx to the lungs. The infectivity of the pneumococcus for man is relatively low; attendants of cases rarely develop pneumonia, but they frequently become carriers of the organism infecting the patient; epidemics are rare and occur usually in crowded communities of individuals with a decreased resistance, as may be found among soldiers in war-time.

TABLE VII

Mortality Rate in Relation to the Type of Infecting Pneumococcus

(After Finland and Brown, 1939)

Type of pneumococcus	With serum			Without serum		
	Cases	Deaths	Deaths per cent.	Cases	Deaths	Deaths per cent.
I	459	85	19	289	155	40
II	234	55	24	196	83	42
V	81	8	10	234	97	41
VII	79	9	12	160	47	29

An alternative theory of the mode of infection is that members of the heterogeneous group IV already present in the throat change in some way to types I and II, and so give rise to an endogenous type of infection. Griffith has found that by injecting a mouse with a live rough culture of one type (I), together with a killed smooth culture of another type (II), he could recover a smooth living organism of the type II. The possibility of infection resulting from such a biological change in type, however, appears less probable than the theory of exogenous infection.

Pathogenesis. After the causative organisms have been implanted in the throat of a susceptible person by droplet infection they pass to the lung, but the precise mode of spread has not been definitely determined. Three routes are probable: (*a*) A surface spread along the air-passages, (*b*) *viâ* the peritracheal and peribronchial lymphatics, and (*c*) by the blood-stream. Spread either directly by the air-passages or by the blood-stream is not indicated by either clinical or experimental observations which suggest spread by the lymphatics. Various stages of the disease process have been examined in monkeys after intratracheal inoculation of virulent pneumococci. The findings indicate that the pneumococcus penetrates the bronchial or tracheal mucosa and passes to the root of the lung by lymphatic spread. Rapid invasion of the lung tissue occurs, resulting in consolidation and the development of clinical signs.

The Response of the Patient. Pneumonia tends to run a characteristic course which is readily recognized by the clinician. The severity of the infection is dependent largely on the type of the infecting pneumococcus and the age and general condition of the patient; the mortality rate is highest in patients over 35, while type III pneumonias are more frequently fatal than type I and II infections. The introduction of serum-therapy has had a decided beneficial influence on some types of infection, in particular types I and II but not III, while the sulphonamides are effective against all types.

Criteria, indicating the reaction of the patient to the infection and the probable prognosis, were admirably analysed by Cruickshank in the Milroy Lectures of 1933 under the following headings:—

(1) **Temperature.** In all cases there is a sharp rise of temperature. In type I infections the level tends to be high throughout the infection; whereas in type II cases there is a gradual fall from the initial high level. Fall by crisis is more frequent in type I than in type II infections and occurs about the sixth day; the fall is accelerated by treatment with the homologous serum or some active chemotherapeutic agent.

(2) **Leucocyte Count.** Infection is accompanied by a definite leucocytosis and the mortality rate appears to vary inversely with the response. When the count is relatively low, under 10,000 per cubic millimetre, the fatality rate is high. The

leucocyte count thus constitutes a particularly important prognostic factor. It may also provide a useful indication of the presence of complications.

(3) **Bacteræmia.** Pneumococci can frequently be isolated from the blood-stream in cases of pneumonia even in the initial stages of infection, and their presence may have an important prognostic significance according to the type of the infecting organism. In the absence of serum-therapy, bacteræmia is present in about one-third of type I cases and about 40 per cent. of type II cases. Bacteræmia has a grave prognostic significance; in type I cases, not serum-treated, the mortality rate has been found to be as high as 80 per cent., while figures, collected in America and Glasgow, indicated that in type III infections the mortality rate of cases with a positive blood culture was 100 per cent. Daily blood-cultures may be required as pneumococci may not be demonstrated in the blood-stream until the fourth or fifth day of the disease (Table VIII).

TABLE VIII

Influence of Bacterœmia on the Death-rate in Lobar Pneumonia

(After Finland and Brown, 1939)

Type of pneumococcus	Without serum therapy					
	Blood culture positive			Blood culture sterile		
	Cases	Deaths	Deaths per cent.	Cases	Deaths	Deaths per cent.
I	140	109	78	245	46	19
II	74	56	76	122	27	22
V	84	67	79	111	17	15
VII	32	28	88	102	16	16

(4) **Toxæmia.** The presence of toxæmia is detected by clinical observations, based on the appearance of delirium, prostration, incontinence, etc. It has been suggested that the generalization of the S.S.S. is concerned with the toxæmic state. It has already been stated that the specific soluble substance or S.S.S. may be

present in the blood-stream and urine. In types II and III pneumonia toxæmia is more severe and S.S.S. has been detected in the urine more frequently than in type I infections, in which the mortality rate is also lower. The amount of S.S.S. elaborated appears to have a great bearing on the course of infection. The type I pneumococcus forms little S.S.S., but is highly invasive; in type I pneumonia the patient's response is usually brisk, and the temperature falls by crisis, little toxæmia being present. Type II pneumococcus produces more S.S.S. and gives rise to a more severe type of infection with more toxæmia. Type III organisms produce most S.S.S. and give rise to a most severe and often fatal form of infection. It has been found that the S.S.S. combines with the antibodies and so prevents the sensitization and subsequent phagocytosis of the pneumococci. It is, however, uncertain whether it exercises a directly toxic action *per se*; no convincing evidence of this has yet been obtained.

Other Pneumococcal Infections

Broncho-pneumonia. Lobular or broncho-pneumonia is a condition which tends to occur when the individual resistance has been lowered. It is thus not uncommonly found during the winter months as a sequel to such debilitating diseases as influenza, measles and whooping cough in the very young and the old. Its pathogenesis appears to be quite different from that of the lobar type, and it may be caused by a large variety of organisms, particularly pneumococci, hæmolytic streptococci and the influenza bacilli. Many observers have found that, when caused by the pneumococcus, in 75–90 per cent. of cases the causative organism is a member of the heterogeneous group IV. This suggests that the infection is endogenous; *i.e.*, owing to the decreased resistance of the individual the relatively non-pathogenic pneumococci, normally present in the naso-pharynx, become actively pathogenic. The infection in these cases spreads directly along the bronchial mucosa, by which the anatomical distribution of the lesions is determined. The mortality rate is high and is largely dependent on decreased resistance resulting from the primary infection as well as the age and physical condition of the patient; the rate is highest in the very young and the very old.

Empyema. Pneumococcal empyema is almost invariably a complication of pneumonia. It occurs most commonly in type I infections (55–85 per cent.) in which serum-therapy has not been carried out; the striking frequency in type I infections is considered to be a manifestation of the invasiveness of this particular type of pneumococcus.

Otitis Media and Meningitis. These conditions occur not infrequently in children, and may be either primary or secondary to an attack of pneumonia. Primary infection occurs directly from the mouth and members of group IV are usually the infecting organisms.

Peritonitis. Primary pneumococcal peritonitis is sometimes encountered in children. Infection is thought to occur by direct spread *viâ* the Fallopian tube from the vulva.

DIAGNOSIS. The material sent for examination in pneumococcal infections is usually pus or sputum. The identification not only of the organism but also of the type is commonly desired, and the following scheme of examination is recommended :—

(1) Examination of smears by Gram's method and perhaps also for the presence of capsules.

(2) Culture on blood-agar and in broth. If the culture is pure the broth can be used for bile-solubility and agglutination. The blood-agar culture will give the typical colonial appearance and α hæmolysis ; if growth is mixed, pick-off separate colonies from the blood-agar plate and sub-culture.

(3) Inoculation of inulin.

(4) Bile-solubility test.

(5) Serological tests. Agglutination is generally carried out by testing the organism against type sera, either by the ordinary macroscopic method, using a pure culture, or by one of the recently introduced rapid methods.

The introduction of serum-therapy in pneumonia demanded the rapid typing of the causative organism and several satisfactory slide methods have been introduced. One useful method originally described by Neufeld and modified by Armstrong does not depend upon agglutination. In this, small portions of sputum are mixed on a slide with a drop of each type serum, and each preparation is then covered with a cover-glass. Microscopical examination is performed after a few minutes' interval, the cocci are swollen and present a ground-glass appearance in the presence of the homologous serum ; this is termed the "Quellung" phenomenon. Much experience is, however, required before reliable results can be obtained. The typing of the pneumococcus is no longer a matter of urgency as the sulphonamides and penicillin, which have almost completely replaced serum-therapy, are effective against all types of pneumococcus.

(6) Animal inoculation. Samples of pneumonic sputum after being well washed and emulsified in saline are injected intraperitoneally into mice; after 4–6 hours a definite peritoneal exudate forms and is collected. Staining for capsules and agglutination tests, macroscopic or by the slide method, are then carried out. Precipitation tests, using the supernatant fluid, may also be employed, particularly when heavy contamination of the original specimen is present.

The differentiation of the pneumococcus from *Strept. viridans* is sometimes difficult; the differences usually found are given in Table IX.

Certain variations may be encountered, *e.g.*, some strains of *Strept. viridans* may produce a general turbidity in broth and/or ferment inulin. The crucial test is bile-solubility; all pneumococci must be bile-soluble, whereas this property is never possessed by *Strept. viridans*. A positive agglutination reaction with a type serum is, of course, conclusive in the case of the pneumococcus.

Specific Prophylaxis. Active immunization by vaccines is not called for in ordinary civil practice, but it has been employed in special circumstances when the incidence of pneumonia has reached epidemic proportions. Lister (1917) carried out an extensive campaign at the Rand mines among the native workers, in whom alarming epidemics developed. Vaccines, prepared from the three common types, were injected intravenously and later

TABLE IX

The Differentiation of the Pneumococcus from Strept. viridans

Test	Pneumococcus	*Strept. viridans*
Morphology	Diplococci	Usually chain-formation
Capsules	$+^{ve}$	Not present
Broth	Turbidity	Usually a granular deposit.
Blood-agar	α hæmolysis and characteristic colonies.	α hæmolysis
Bile-solubility	$+^{ve}$	$-^{ve}$
Inulin fermentation	Usually $+^{ve}$	Usually $-^{ve}$
Agglutination	$+^{ve}$ (with type sera)	$-^{ve}$

subcutaneously in three large doses. There was subsequently a marked decrease in the incidence of pneumonia due to these types among the natives. Active immunization was also employed at various American Army camps during the war with some success. The results, while difficult to assess, indicate that, following the use of vaccines, there is an increased resistance against the homologous types.

Felton has recently introduced a preparation of the specific capsular polysaccharide for active immunization against types I and II pneumococci. The results, while encouraging, are not yet conclusive.

Recent investigations suggest that, contrary to the accepted view, recovery from pneumonia is accompanied by an increased resistance of short duration to further infection with the homologous type ; when second attacks occur they are usually due to infection with one of the other types. The precise mechanism of the immunity response in pneumococcal infections is, however, still undetermined.

Therapy. The application of *specific therapy* to pneumonia has been mainly due to the efforts of certain American workers during the past 15 years, particularly to the introduction by Felton of a method of refining and concentrating the serum antibodies. Extensive investigations have been made and several facts have been definitely established. Types I and II pneumonia have responded well to serum-therapy, but in type III cases the serum appears to be of little value. In group IV cases serum has also given valuable results, particularly in infection with types V, VII and VIII. These observations emphasize the necessity for the rapid identification of the type of infecting pneumococcus in cases of pneumonia when serum-therapy is being instituted.

The serum must be administered early, intravenously and in adequate doses. The results are often striking, as shown by a diminution in the severity of the symptoms and a reduction in temperature, pulse rate and respiratory rate. The course of the disease is shortened, the mortality rate lowered and the incidence of complication reduced. The main action of the serum appears to be the promotion of phagocytosis. In order to save delay some workers employ a polyvalent serum, containing types I and II antibodies, at once. The type of the infecting organism is then determined, and if this proves to be either I or II the homologous serum is then administered. It is considered that some of the failures of serum-therapy can be explained by the late administration of the serum and also by the faulty typing of the infecting organism. Figures recently published in America show that in 160 cases of type I pneumonia treated during the first 24 hours of the disease the mortality was only 5 per cent.

The serum dosage has varied in different countries. In America, where serum has been extensively employed, larger doses are used than in Britain, where an initial dose of 10–20,000 units is frequently given, followed by similar amounts every 8–12 hours until the temperature falls below 102° F. The total amount employed by different workers has been 100,000, 75,000 and 36,000 units. In some cases, particularly when the serum is given after the fourth day and bacteræmia is present, doses of 40–100,000 units have been given every 8 hours. The sufficiency of the serum-dosage may be estimated either by attention to the clinical condition of the patient and by specific tests such as the presence of homologous agglutinins in the serum or the allergic reaction described by Francis. Francis (1933) introduced a useful index for serum-therapy; a small amount of the homologous polysaccharide is injected intradermally, if an immediate positive reaction, as evidenced by a local erythema, occurs, serum-therapy need not be continued. Other workers have controlled the administration of serum by the presence of homologous agglutinins in the serum; when a marked agglutination reaction occurs serum-therapy is discontinued.

Antipneumococcal serum is prepared by the intravenous injection of young killed cocci into the horse; after a prolonged period of immunization, perhaps as long as 9 to 15 months, a potent serum is usually obtained. The antibody is concentrated, but this process is rendered difficult by the fact that the antibody is associated with the water-insoluble protein. Much loss occurs during concentration and the final product is somewhat expensive. It also gives rise not infrequently to unpleasant reactions.

Standardization of the serum has been rather unsatisfactory, and it is only recently that an official standard has been accepted. A *unit* has been defined by Felton as the amount of serum which will protect mice against one million lethal doses of a culture when serum and culture are given simultaneously. In this country a dry standard serum, tested against Felton's serum by the above method, is employed as a basis of standardization. This serum has recently been adopted by the League of Nations Commission on Biological Standardization as the official international standard. In the case of type I antiserum the activity contained in 0·0886 mg. of the serum is defined as one international unit; with type II antiserum one international unit is defined as the activity contained in 0·0894 mg. of the serum.

A further development in serotherapy has been the preparation of antisera in rabbits. This serum has many advantages over that prepared in the horse: a higher titre can be obtained, the preparation of antiserum against the rarer types becomes an economic proposition, and also serum therapy can be carried out in patients hypersensitive to horse protein.

Chemotherapy. The serum-therapy of pneumonia has never been popular in this country and now it has been almost entirely superseded by chemotherapy. The sulphonamides, in particular recent products such as sulphathiazole, sulphadiazine and sulphamezathine, give excellent results; sulphanilamide preparations are, however, ineffective. Cases of lobar pneumonia respond well to a routine course; regular and adequate dosage is essential and a copious intake of fluids must be rigorously enforced. Penicillin is also effective in pneumonia but, because of difficulties of administration and the excellent results given by the sulphonamides, it is usually reserved for sulphonamide-resistant cases. Penicillin given intrathecally as well as intramuscularly, with or without a routine course of a sulphonamide, has given good results in the treatment of pneumococcal meningitis (*cf.* Chapter VII).

CHAPTER XVII

NEISSERIA : GONORRHŒA : MENINGITIS

THE main features of the genus *Neisseria* are : Gram-negative cocci usually arranged in pairs, growing poorly on the simple media and strict parasites in man.

A number of important organisms belong to this genus, the name of which has been taken from Neisser, who described the first member in 1879 when he observed the gonococcus in the urethral pus of patients with gonorrhœa. This organism was subsequently cultivated on artificial media and its ætiological relationship with gonorrhœa conclusively established. A few years later Weichselbaum (1887) isolated the meningococcus from the cerebro-spinal fluid of cerebro-spinal meningitis cases. Other members were later obtained from the nasopharynx of healthy individuals ; Pfeiffer (1896) described the *Micrococcus catarrhalis* and V. Lingelsheim (1906) identified several forms : *M. pharyngis siccus*, *M. pharyngis cinercus* and *M. pharyngis flavus*. It is interesting to note that, with the exception of the gonococcus, the various species exist almost solely in the nasopharynx of healthy and diseased individuals ; the meningococcus is, however, also found in the cerebro-spinal fluid of cases of meningococcal meningitis.

GONOCOCCUS OR NEISSERIA GONORRHŒÆ

Morphology. The gonococcus is a spherical or oval coccus, 0·6 × 0·8μ, usually arranged in pairs with the adjacent sides flat or concave so that between them is a small oval unstained area, the whole resembling two beans placed side by side ; the long axis of the gonococcus is at right angles to the axis joining the two cocci. This arrangement is almost invariably found during growth in the body, when the majority of the cocci are frequently situated inside the leucocytes. Under the conditions of artificial cultivation the cocci tend to become spherical, and varied arrangements may be found, such as pairs, small groups and single forms. It is non-motile, and does not form either spores or a demonstrable capsule. The gonococcus is readily stained by the ordinary aniline dyes, but it is decolorized in Gram's method, *i.e.*, it is Gram-negative

Cultural Characteristics. The gonococcus is not easy to cultivate. Growth is very slight or absent on most simple media ; enrichment with some animal protein such as blood, serum or ascitic fluid provides a satisfactory medium which in all cases must be freshly prepared and moist. The optimum temperature is 37° C., and the temperature range is small. Aerobic conditions are required, but on first isolation growth is frequently improved by the presence of 10 per cent. CO_2 and water vapour. The optimum reaction of the media is pH 7·4–7·6, but growth may occur over a wider range provided the medium and atmospheric conditions are satisfactory.

In *fluid media, e.g.*, serum-broth, growth is slow and poor. In 24–48 hours a slight granular deposit forms with little or no general turbidity.

On *solid media* the appearance of colonies, on first isolation, is sometimes delayed for 48 hours. They are small, 0·6–1 mm. in diameter, moist, greyish-white and semi-transparent, tending to become more opaque and somewhat granular on further incubation. In some cases the edges of the colonies may exhibit marked scalloping, while the colonial appearance may be extremely variable.

Biochemical Activity. The gonococcus exhibits very little biochemical activity. It, however, ferments some carbohydrates with the production of acid, but not gas. For identification purposes glucose and maltose, in Hiss's serum-water, are frequently utilized, glucose being fermented, but not maltose (see Table XI). No change is produced in litmus-milk.

Resistance. The gonococcus is readily destroyed by bactericidal agents. It is susceptible to desiccation and is killed by heat at 55° C. in 5 minutes ; silver compounds have a strong bactericidal action, and they are extensively used in the therapy of gonococcal infections.

Owing to the low degree of resistance and the tendency to autolysis the conservation of stock-cultures is difficult. Cultures should be prepared on moist suitable media, incubated at 37° C., and sub-cultured every 7–10 days, or dried *in vacuo*.

Serology Agglutination, agglutination-absorption and complement fixation have been employed by many workers to sub-group the gonococci. The results, however, have been far from conclusive. A large variety of sub-groups appears to be present, but the differentiation of many of these is not sharply defined. In spite of this many workers have been able by agglutination to make a primary sub-division into two main serological groups (types I and II). Most of the strains isolated from acute cases fall into type I, whereas strains from chronic cases tend to belong to type II. It is considered that the transition of type I strains to type II is a gradual process resulting in the formation of numerous sub-types, which are responsible for the frequent irregularities.

The gonococcus appears to have a complex antigenic structure. Fractionation tests suggest that a group-polysaccharide and a group nucleoprotein are present together with certain undefined type-specific factors.

Pathogenicity. The gonococcus gives rise to spontaneous infection only in man, gaining entrance by either the genito-urinary mucosa or the conjunctiva and producing eventually a purulent infection.

Its offensive weapons have been incompletely examined, as laboratory animals are refractory to infection; gonorrhœa has, however, been reproduced by the inoculation of the gonococcus into the human urethra. The intraperitoneal injection of either a living or a lysed culture into guinea-pigs and rabbits gives rise to a definite, but non-specific, disturbance which may end fatally. The responsible substance is probably of the nature of an " endotoxin ".

Gonorrhœa

Gonorrhœa is an ancient disease, in which infection is usually conveyed directly by sexual intercourse. Following implantation of the gonococcus on the urethral mucosa there is a variable incubation period of 2–8 days, after which a mucoid discharge, which rapidly becomes purulent, appears. The organism penetrates between the columnar cells of the mucous membrane, and inflammatory changes occur around the mucous glands and in the submucosa. If adequate treatment is not undertaken immediately, the infection may spread, either by continuity along adjacent surfaces or through dissemination by the lymphatic system or blood-stream.

Spread by continuity is a frequent event. Lesions of the posterior urethra, prostate, epididymis and testicles are not uncommon in the male. In the female the infection passes to the vagina, which in adults appears to be relatively resistant, and inflammatory changes are often produced in Bartholin's gland and the cervix of the uterus. Spread along the endometrium is not common, but when it occurs infection may pass to the ovaries and peritoneum by the Fallopian tubes, where salpingitis or pyosalpinx may be produced. The rectum may also be involved, in which event a purulent proctitis generally develops.

A generalized infection is more frequent in males than females. The gonococcus enters the blood-stream, from which it may be cultured, and tends to localize in joints and the cardiac endothelium. Gonococcal arthritis was a relatively common complication; it is considered by some workers to occur in about 1 per cent. of all cases of gonorrhœa. Endocarditis occurs less frequently but is a more serious complication. In a few isolated cases metastatic lesions are found in the pleural cavity, peritoneum and meninges.

Once established the infection is particularly resistant to treatment, and a complete cure is only obtained with difficulty. In the absence of satisfactory treatment, the organisms may remain viable in the tissues for many years and may not be detected by routine bacteriological examination. After the acute symptoms subside the disease takes a chronic course, in which event the urethral discharge may be scanty. These cases are, nevertheless, capable of infecting others, and any subsequent temporary lowering of the general resistance may be followed by an acute exacerbation of the infection.

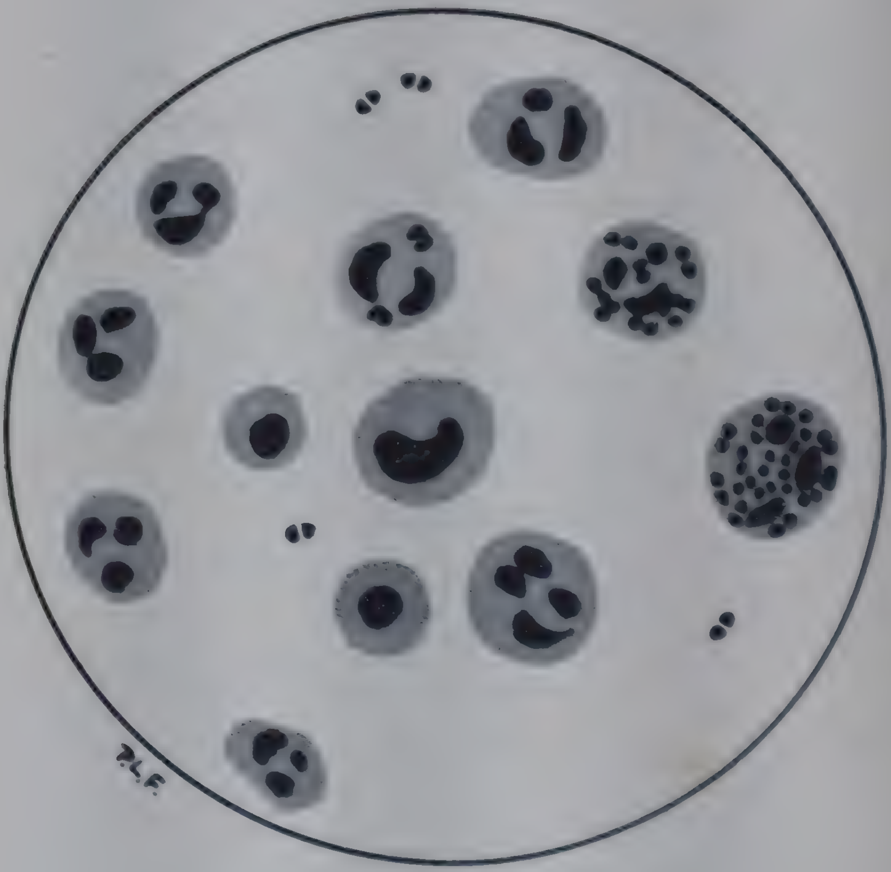
In children the gonococcus may give rise to a vulvo-vaginitis; in these cases the infection is usually conveyed by contaminated towels, clothes, linen, etc. The infection tends to remain localized in the vagina, which appears to be more susceptible than in adults, but occasionally it may spread to the peritoneum.

When the mother is suffering from gonorrhœa, infants may acquire, during delivery, a purulent infection of the conjunctiva, termed "ophthalmia neonatorum". This condition has decreased considerably as a result of the introduction by Credé of the prophylactic instillation of some silver compound into the eye immediately after birth. This procedure has been widely adopted as a routine measure.

Diagnosis. Three main methods may be employed in the diagnosis of gonorrhœa :—

(1) Microscopical examination of smears.
(2) Preparation of cultures.
(3) Serological tests.

PLATE I



Gonococci in film of pus from case of acute gonorrhœa (stained by Gram's method). × 1000.

[To face p. 211.

1. Microscopy. In the majority of instances the microscopical examination of smears is the sole method used. In males smears of pus prepared from the discharge in acute cases, and after prostatic massage in the chronic forms, are stained by Gram's method. The gonococci are Gram-negative, and during the early stages of infection may be mainly extracellular, but later they present a more characteristic appearance. Many of the pus cells are free from bacteria, but some contain large numbers of the Gram-negative diplococci (Plate I). In the chronic form some cocci may be extracellular.

In females smears collected from the urethra, cervix and any exudate from the ducts of Bartholin's glands frequently prove satisfactory. An appearance similar to that in the acute male cases is found. In chronic cases smears, particularly those taken from the vagina, are unsatisfactory, because considerable numbers of other organisms are usually present and the identification of the gonococcus is impossible.

A possible fallacy of this method of examination is that Gram-positive cocci, which are situated intracellularly, may be decolorized and appear as Gram-negative cocci. This is a rare event, but such a possibility must always be excluded, particularly in chronic cases.

2. Preparation of Cultures. The ideal diagnostic measure is the isolation of the gonococcus in pure culture from the tissues. This can only be satisfactorily carried out when the laboratory is in close connection with the clinic. The gonococcus is difficult to grow, and the collection of material and the selection of the medium both require close attention. In the female cultures should be prepared from material collected from both the urethra and cervix. A satisfactory medium is chocolate-agar, which should be incubated in an atmosphere containing 10 per cent. CO_2. A useful method of detecting gonococcal colonies in mixed cultures is the *Oxidase Reaction*, which depends on the production of an oxidizing mechanism by this organism. Plate-cultures are made on heated blood-agar and, after 48 hours' incubation, 0·5–1 per cent. tetramethyl-*p*-phenylene-diamine solution is poured on; colonies of gonococci develop a black discoloration after an initial purple stage, *i.e.*, they give a positive oxidase reaction. If subcultures are required they should be made without delay, as the organisms die soon after the addition of the solution.

3. Serological Tests. Agglutination has proved unreliable as a diagnostic measure, but the application of complement fixation has recently been re-examined and a satisfactory method is now available. Price (1933), employing as the antigen organisms extracted with NaOH, made a detailed investigation of the reaction. He found *inter alia* that (i.) specific antibodies could not usually be demonstrated before the second or third week; (ii.) the results depended on the anatomical distribution of the disease, since when this was confined to the anterior urethra in the male or urethra in the female negative results were obtained; (iii.) the type of infection, *i.e.*, whether open with free drainage, or closed without drainage, influenced the result; the former cases tended to be negative; (iv.) cases treated in the early stages seldom gave a positive reading. A negative result does not therefore indicate freedom from infection. A persistent negative result after treatment of a previously positive case denotes a cure. Complement-fixation tests have given excellent results in chronic infections in females, which are extremely difficult to detect by the other methods of examination.

Murray (1933) employed the three methods in the examination of 500 consecutive cases of gonorrhœa in females and compared their diagnostic value. The results are given in Table X.

TABLE X

Diagnosis of Gonorrhœa in the Female. (After Murray)

Method	Positive	Negative
	Number per cent.	Number per cent.
Cultures	334 (66·8)	166 (33·2)
Complement fixation . .	293 (58·6)	207 (41·4)
Smears (urethra and cervix) .	227 (45·4)	273 (54·6)

It is seen that cultural methods gave the best results, but unfortunately the application of this method in routine practice has definite limitations (*vide supra*). It is interesting to note that the examination of smears gave disappointing results and was definitely inferior to the other two methods.

Prophylaxis. Specific prophylactic measures are of no value in preventing gonococcal infection. This is not unexpected, as little immunity appears to result from an actual attack of gonorrhœa, second infection being common. In some so-called second attacks, however, this may be merely a " flare-up " of the primary infection, which, instead of clearing up, has become quiescent.

Avoidance of infection and the local use of bactericidal agents are the most valuable methods of prevention. As previously stated, the method introduced by Credé has been very successful in the prevention of ophthalmia neonatorum.

Therapy. Specific therapy by means of vaccines is employed in some clinics. The main value of vaccines is probably in the treatment of complications; they tend to prevent complications such as arthritis and endocarditis, resulting from a generalization of infection. Vaccines are also used to flare up a latent focus and, by thus exposing it, enable it to be treated with the various chemical agents. Vaccines may stimulate the production of antibodies and so produce a positive complement-fixation test, but, in the absence of infection, the test soon becomes negative. The interpretation of a positive reaction in individuals who have recently received vaccines may require very careful consideration, as in such cases a positive reaction does not necessarily indicate the presence of infection.

Chemotherapy is now the standard form of treatment for gonococcal infections. The sulphonamides have been extensively and successfully used, but in recent years there has been an increasing number of failures due to the development of resistant strains of the gonococcus. The organism is, however, extremely sensitive to penicillin and this antibiotic is now the drug of choice in the treatment of gonorrhœa. The optimum dosage and duration of treatment have not yet been determined, but cases have responded well to a total dosage of 150–200,000 units. This amount has been given either in multiple injections at 2- or 3-hourly intervals or in a single dose with the penicillin incorporated in some slow-release vehicle, such as peanut oil and beeswax. Failures of treatment are uncommon; when they occur the response to a second course of penicillin is usually good.

MENINGOCOCCUS OR NEISSERIA MENINGITIDIS (INTRACELLULARIS)

N. intracellularis was the name adopted for the meningococcus by the American Society of Bacteriologists; *N. meningitidis* appears to be more satisfactory, as the intracellular position of the organism is probably more a matter of chance than its relationship to epidemic meningitis in man.

Morphology. The morphological appearance of the meningococcus is practically identical with that presented by the gonococcus. A typical picture is usually found when present in the body—Gram-negative diplococcus with flattened adjacent sides and frequently in an intracellular position. In artificial cultivation this arrangement is frequently lost, single forms, pairs or groups are arranged irregularly and much variation may be found in the size of the individual cocci. In old cultures there is usually great variation in staining, and so-called "ghost" forms may be present. The meningococcus is non-motile and does not form spores or a demonstrable capsule.

Cultural Characteristics. The meningococcus is usually easier to grow than the gonococcus. On first isolation enriched media, such as blood-agar or serum-agar, are necessary, but, after several subcultures, growth may occur on the ordinary media ; it is, however, never profuse.

The optimum temperature is 37° C., and growth may be obtained over a range of 30°–40° C. It is a strict aerobe, there being no growth under strict anaerobic conditions. It has recently been found that growth is more marked in the presence of 10 per cent. CO_2 ; this is particularly important in the isolation of the organism from cerebrospinal fluid on a blood-agar plate. The most favourable reaction of the media is pH 7·4–7·6.

On *solid media,* in 24–48 hours, the meningococcus frequently forms characteristic colonies which are smooth, translucent and round (1–2 mm. diameter) with a regular edge and a bluish-grey tinge. They are readily picked off and emulsify without difficulty. On further incubation the colonies become opaque, slightly yellowish and may be differentiated into a central granular area with a distinct peripheral zone.

In *fluid media* growth occurs as a slight general turbidity with a granular deposit.

Biochemical Activity. The meningococcus displays a limited biochemical activity. Gelatin is not liquefied and indole is not produced. Some carbohydrates are fermented with the production of acid, but not gas. In practice maltose and glucose are employed in Hiss's serum-water ; both are fermented (see Table XI). There is no change in litmus-milk.

Resistance. The meningococcus is readily destroyed by bactericidal agents. It is killed by heat at 55° C. in 5 minutes and 1 per cent. phenol in 1 minute. Methylene-blue has a specific inhibitory action in high dilutions. Marked autolysis takes place after incubation at 37° C. for several days ; this effect is considered to be due to the action of an intracellular enzyme.

Stock cultures are difficult to conserve owing to the marked readiness with which the organism dies out. Survival is longest at 37° C., and on Dorset's egg medium, as on this medium growth is rather poor and autolytic changes are delayed. The use of semi-solid blood-agar has also given satisfactory results ; drying *in vacuo* is satisfactory.

Serology. There are definite indications that the meningococcus has a complex antigenic structure ; a group polysaccharide, a group nucleoprotein and a type-specific polysaccharide have all been isolated. It is therefore not surprising that the results obtained by serological tests are frequently irregular and are not fully understood.

Agglutination tests have been extensively used in the study of the meningococcus, and it was soon found that the meningococci were not serologically homogeneous. Dopter (1909) isolated, from cases of meningitis in Paris, strains which differed serologically from the type usually encountered ; these irregular strains were classified as the " parameningococcus ". Further advance was made when Gordon (1915) found that cocci isolated from cases of meningitis in British and Canadian troops during the First Great War fell into four groups, which he styled types I, II, III and IV. Subsequent work showed that these four groups did not embrace all meningococci, and also that marked cross-agglutination occurred. Types I and III and types II and IV exhibited marked interaction, and consequently some workers considered that the meningococcus should be primarily sub-divided into two main groups ; group I embraces Gordon's types I and III and group II types II and IV. The heterogeneous non-agglutinable strains are usually isolated during inter-epidemic periods, particularly from the naso-pharynx.

Toxin Production. There is no doubt that the meningococcus forms a toxin which possesses the characteristics of the so-called " endotoxins " ; it is relatively heat stable, but, being difficult to demonstrate, its properties have not been determined with any exactitude. The endotoxin has been liberated from the organisms by varied methods, such as autolysis and treatment with weak caustic soda or bile salts. On intraperitoneal inoculation of large doses into the rabbit, guinea-pig or mouse death results from toxæmia in 2–4 days. This action is more marked with lysed cultures than with the living cocci, and the same endotoxin is apparently produced by all types of the organism.

Ferry and his colleagues in 1931 claimed that they had obtained from the meningococcus a substance having the properties of an "extracellular" toxin. This toxin was present after growth in broth for 4–6 days and was specific for each type. It produced a local reaction on intradermal inoculation into children, and corresponding antitoxins developed on injection into animals. Ferry considered that these toxins were largely responsible for the symptoms of cerebrospinal meningitis; the intrathecal inoculation of the toxin into monkeys gave rise to symptoms similar to those resulting from the injection of the living cocci. These symptoms were definitely modified by adding the corresponding antitoxin to the toxin before injection. As a result of these observations Ferry considered that the most suitable therapeutic antiserum was one containing antitoxins.

Some of these observations have been confirmed, but the actual production of an exotoxin by the meningococcus is not yet generally accepted. The rapid autolysis of the cocci and the subsequent diffusion of the liberated coccal protein have not been excluded as participants in the reaction by the supporters of this theory.

Pathogenicity. The meningococcus is a human pathogen and is responsible for cerebrospinal meningitis or spotted fever as it was formerly termed. The organism may also give rise to a condition known as chronic meningococcal septicæmia in which the meninges are not usually involved, and rarely to a purulent conjunctivitis. A meningitis may be produced in monkeys if pure cultures are injected intrathecally.

Cerebrospinal Meningitis

In man the pathogenesis of cerebrospinal meningitis is uncertain; two important factors are, however, concerned—(1) the virulence of the organism and (2) the susceptibility of the host. The condition is common in wartime and it was widespread in this country during 1940 and 1941.

When major outbreaks appear they tend to be localized and to occur mainly in crowded communities such as schools and barracks. The outbreaks are not epidemic in the strict sense, as sporadic cases only are encountered even in apparently susceptible communities. The meningococcus is introduced by droplet infection from other cases or, more usually, carriers into the naso-pharynx of the host, where it settles and multiplies. The carrier-rate is naturally highest where overcrowding is present. The majority of individuals remain unaffected, but in some a rhino-pharyngitis results ; this usually resolves without causing the patient any appreciable discomfort. These individuals become temporary carriers ; the meningococci usually disappear in 1–2 months, but during this time the organism may be widely disseminated, particularly in crowded communities. Observations made by independent workers have shown that the carrier-rate in a community may be as high as 50 per cent. At one time it was accepted that a carrier-rate of 20 per cent. or over was an index of an imminent outbreak. It has, however, been recently demonstrated by many workers that, with carrier-rates of 20–50 per cent., even in a closed community, cases of meningitis did not develop. It would consequently appear that the virulence of the organism is an important factor in the development of meningitis. It is therefore interesting to note that during outbreaks of meningitis the responsible strains belong to Group I, whereas strains isolated from the naso-pharynx of carriers tend to be Group II.

Involvement of the meninges occurs in relatively few cases ; the resistance normally offered by the body appears sufficient to prevent the organism, after implantation on the mucosa of the naso-pharynx, from reaching this site. When, however, the defence mechanism is lowered by fatigue and the chance of infection increased by unhygienic surroundings, cases of meningitis tend to develop. It is thus found in soldiers during war-time conditions ; the factors probably responsible are the overcrowding in barracks and the fatigue resulting from the frequently heavy duties involved. Another point is that epidemics usually occur in the winter and spring months, when the general resistance is most likely to be at a low ebb. Cases of meningitis seldom develop from contact with other cases ; infection is transmitted mainly by carriers.

The route taken by the meningococcus in its passage to the meninges has not been definitely established. Some workers maintain that there is an invasion of the blood-stream with subsequent localization of the cocci in the meninges. Evidence considered to support the hæmatogenous route is that blood cultures are sometimes positive in the early stages of the disease, particularly when the petechial rash is present. Others believe that the spread occurs by direct extension along the perineural lymphatics of the olfactory nerve *viâ* the cribriform plate. This route is suggested by the experimental disease in monkeys. According to this theory the cocci appear in the blood after the development of the purulent meningitis, *i.e.*, the bacteræmia is a secondary phenomenon.

Cerebrospinal meningitis usually runs an acute course, the mortality rate, in the absence of serum-therapy or the administration of sulphonamide preparations, is high, usually about 70 per cent. of cases. With the introduction of sulphonamide therapy the mortality-rate has been greatly reduced to some 5–15 per cent. Early diagnosis and intensive administration of the drug are of the greatest importance in obtaining satisfactory results.

Diagnosis. (1) *Cerebrospinal Fluid.* In cases of meningitis the meningococcus can often be detected in the cerebrospinal fluid without much difficulty. The fluid is usually turbid, under greatly increased pressure, and contains a large number of polymorphonuclear leucocytes ; in the early stages these may, however, be scanty. Smears of the centrifuged deposit and cultures should be prepared.

In examining the smears, stained by Gram's method, the presence of Gram-negative diplococci, which are usually intracellular, is noted. In some cases they may be numerous, but in others some difficulty may be experienced in finding them.

Cultures should be made on a suitable medium, such as Loeffler's serum or blood-agar, preferably at the time of lumbar puncture. Growth can, however, be obtained some time after collection in many cases, and when it occurs the organism may be identified by fermentation tests and then typed by means of the agglutination test.

(2) *Blood Culture*, particularly in the early stages of meningitis, is recommended by many workers ; this is not, however, a common routine examination.

(3) *Naso-pharyngeal Swabs.* The meningococcus is often found in the naso-pharynx of both cases and carriers. Much difficulty may, however, be experienced in the isolation and identification of the meningococcus owing to the presence in this region of many bacteria, including other members of the *Neisseria* genus. This examination should, therefore, only be carried out by experienced workers. Swabs are taken by means of a West's swab, which is a curved wire with cotton-wool at one end and contained in a curved glass-tube with open ends. The glass-tube is passed to the back of the mouth and the curved part is pointed towards the naso-pharynx ; the swab is then pushed out and rubbed against the mucous membrane, after which it is withdrawn into the glass-tube, which is then removed from the mouth.

By this method oral contamination is avoided. Cultures should be prepared on blood-agar plates. After incubation for 24 hours, colonies resembling those of the meningococcus are picked off and subcultured. These are examined by smear preparations, biochemical tests and agglutination. The biochemical tests consist of the inoculation of maltose, glucose and sucrose in Hiss's serum-water. The results given by these sugars are reasonably consistent and provide a useful means of differentiating the important members of the genus *Neisseria* (Table XI).

Meningococcal colonies may be identified by means of the oxidase reaction ; they turn black after the addition of the diamine solution (p. 211).

TABLE XI

Fermentation Reactions of Certain Members of the Genus Neisseria

Organism	Glucose	Maltose	Sucrose
N. catarrhalis . . .	–	–	–
Gonococcus . . .	+	–	–
Meningococcus . . .	+	+	–
N. pharyngis . . .	+	+	+–

Prophylaxis. In view of the frequency of the meningococcus in the naso-pharynx of normal individuals, it is now generally accepted that the segregation of carriers is of little value as a routine prophylactic measure ; the swabbing of contacts is therefore seldom necessary. It is, however, important that (1) opportunities for the dissemination of the meningococcus should be restricted by such steps as the reduction of overcrowding and the use of free ventilation, and (2) the resistance of the individual should not be lowered by fatigue or lack of adequate diet.

In special circumstances, such as a severe localized outbreak, chemoprophylaxis with sulphonamides may be useful. This practice should not be adopted as a routine procedure in view of the risk of toxic reactions in the recipient and the possible development of resistant strains of meningococcus.

Therapy. The main therapeutic measures are spinal drainage and the administration of sulphonamide preparations. Serum therapy is now seldom used.

Discordant results have been obtained from the use of serum, which was first introduced by Flexner. The majority of observers have found that the mortality rate is definitely reduced ; before the introduction of serum the mortality rate averaged 70–80 per cent., and after 30 per cent. Two types of sera are now available, the antimeningococcal serum of Flexner and the antitoxin advocated by Ferry. In both cases specific or polyvalent sera are prepared. Antitoxic sera are prepared by the injection of horses with the specific toxins ; their value is, as yet, uncertain, as they have not been employed sufficiently to enable one to pass judgment on their merits. The results obtained by their use in various institutions appear to be encouraging.

Antibacterial serum has been employed extensively. It is prepared by injecting horses intravenously with killed or living cocci. One of the disadvantages of this serum is that there is no accepted standard method of determining its potency. The formation of agglutinins and opsonins and the ability of the serum to neutralize the action of the common endotoxin have been employed as methods of standardization in different laboratories. It is therefore possible that some of the reported failures of serum-therapy might have been due to the use of sera of low potency.

The serum is given intrathecally after the withdrawal of a large amount of cerebrospinal fluid. It should be given as early as possible in doses of approximately 30–60 c.c. for adults and 10–20 c.c. for infants. The serum should be previously warmed to the body-temperature and the amount given must be less than the quantity of cerebrospinal fluid withdrawn. The serum should be given once or twice daily until the symptoms subside. The intrathecal inoculations may be given by lumbar or cisternal puncture and may be supplemented by intravenous injections. The value of the serum is considered to lie in the following activities : (1) neutralization of the endotoxin, (2) the promotion of phagocytosis and (3) lysis of the cocci.

Chemotherapy with sulphonamide preparations has given extremely valuable results in the treatment of meningococcal meningitis ; early, intensive and regular administration has greatly reduced the mortality rate. It was originally thought the greatest effect would be observed when these drugs were given along with antimeningococcal serum, but later observations indicated that the administration of the drugs alone is most effective, particularly if the initial dosage is adequate. It is recommended that the daily dosage for the first few days should be from 3 to 9 gm., according to the age of the patient. All types of the meningococcus appear to be susceptible to chemotherapy with these drugs. In cases of delirium or unconsciousness some soluble form of sulphonamide should be administered intravenously. Penicillin given intrathecally as well as intramuscularly has given good results.

Chronic Meningococcal Septicæmia

Chronic meningococcal septicæmia is a form of meningococcal infection which has recently received much prominence. Although this condition was described during the Great War of 1914–18, and later by various observers, it was generally considered to be uncommon. Many cases have, however, been reported during the recent extensive outbreaks of cerebrospinal meningitis and it would seem that previously the disease had not been widely recognized. Observations indicate that meningococcal septicæmia is particularly prevalent during outbreaks of cerebrospinal meningitis, so that at those times especially the condition should be suspected in all cases of vague pyrexia. Chronic meningococcal septicæmia is characterized by recurrent pyrexia, muscle- and joint-pains and a rash, often petechial, over the limbs and back of the body. The rash disappears in a few days but tends to reappear with the next pyrexial period. The disease, in the absence of sulphonamide therapy, runs a chronic course and may persist for many months. Invasion of the meninges may occur but is not usual.

The diagnosis can only be definitely established by the isolation of the meningococcus from the blood. This is frequently difficult ; cultures should always be collected during a pyrexial period and may have to be repeated several times before a positive result is obtained.

Treatment with sulphonamide derivatives is very effective ; the symptoms usually disappear within 24–48 hours.

Neisseria (Micrococcus) catarrhalis

N. catarrhalis is a Gram-negative coccus usually arranged in pairs or tetrads. On first isolation from the tissues it grows rather poorly on simple media which should be enriched with serum or blood. After several subcultures it grows readily on the ordinary media. On agar variation of the colonial appearance is found ; one common type is definitely roughish and another is smooth, resembling that formed by the meningococcus. In broth growth occurs as a granular deposit. The optimum temperature is 37° C., but multiplication may take place at 25° C. Its biochemical activities are very limited, carbohydrates are not fermented.

N. catarrhalis is normally present in the naso-pharynx of man, but it has a low pathogenicity for man and animals. It is usually found as a secondary invader in lesions of the upper respiratory tract, and is therefore usually included in vaccines prepared to counter colds or influenza. Its main claim to importance is that it must be excluded in the examination of naso-pharyngeal swabs for the meningococcus.

Neisseria pharyngis (Micrococcus pharyngis flavus and siccus group)

A number of Gram-negative cocci, some forming yellowish colonies on agar, has been isolated from the upper respiratory tract of man. These have been incompletely investigated, but they have been tentatively grouped into different groups, according to their cultural and fermentation reactions ; some three or six groups have been described by different workers. This subgrouping is unsatisfactory, and the various cocci are probably best considered tentatively with the other indefinite Gram-negative cocci found in this situation as one heterogeneous group, *N. pharyngis*. They are normally present in the upper respiratory tract, where they may exercise a mild pathogenic action as secondary invaders ; they must be differentiated from the meningococcus, and they consequently interfere with the isolation of the latter organism from the naso-pharynx.

CHAPTER XVIII

ORGANISMS OF THE COLI-TYPHOID-DYSENTERY GROUP; GENUS BACTERIUM

THE main generic characteristics of this group are: plump rods, without spores, Gram-negative, motile by means of peritrichate flagella, or non-motile, liquefying gelatin very slowly or not at all; usually showing marked power to ferment carbohydrates, frequently with the production of gas; typically intestinal parasites of man and higher animals, although several species may be widely distributed in nature; many are pathogenic to man and animals.

A considerable number of species is included in this genus. The first member to be described was a pathogenic species, the typhoid bacillus or *Bact. typhosum*, which was observed in the spleen and mesenteric glands of a fatal case of enteric fever by Eberth in 1880. It was subsequently isolated in pure culture by Gaffky in 1884. *Bact. coli*, a relatively non-pathogenic member, was next isolated by Escherich in 1885 from normal fæces. Since then a large number of distinct species, both pathogenic and non-pathogenic, have been isolated from various sources, but mainly from the intestinal tract of man and animals. In some cases the differentiation of the species has been relatively simple, but in other instances much difficulty has been experienced in establishing the individual identity of a species. Many schemes for the sub-division of this genus have been introduced, but none has received universal recognition. However, a widely employed and simple method of primary classification has been based on the power of the different organisms to ferment lactose; as a general rule pathogenic species do not ferment lactose and consequently are frequently referred to as the "non-lactose-fermenters"; the non-pathogenic members on the other hand usually possess this property and are often termed the "lactose-fermenters".

The limits of this group are rather ill-defined, and the tendency was at one time to include all forms possessing some of the generic characters. This was unsatisfactory and detracted considerably from the taxonomic value of the generic title. In recent years the practice has, however, been in the reverse direction, *i.e.*, a tendency to place associated organisms into separate genera. For example, the cocco-bacilli associated with undulant fever and contagious abortion had been tentatively placed in this genus, but they have recently been separated from this group and arranged in a separate class with the generic name "Brucella". The arrangement and terminology of the sub-genera have been confused by the introduction in the genus *Bacterium* of such terms as "Eberthella," "Shigella" and "Escherichia". There is little to support such a haphazard system of nomenclature, and these terms are seldom used in this country.

It is likely that the present chaotic state will be clarified by the Nomenclature Sub-Committee of the International Society of Microbiology, which, in 1934, issued a scheme in which the serologically related non-lactose-fermenters, pathogenic for man and/or animals, were separated off from the other members of this genus. This group has been given the generic title "Salmonella," which had been tentatively used by earlier workers for various sub-groups of the main genus. The term "Bacterium" is undesirable as a generic title owing to its dual application, but it is apparently to be employed, for the present at least, for the remaining species, which include the coliforms and the various dysentery bacilli.

Morphology and Staining Reactions. The members of this genus cannot be differentiated by their microscopical appearance. The usual form of all is a rod with rounded ends, the average size being about $3 \times 0{\cdot}6\mu$, but marked variation is frequent, coccal and filamentous forms often being present. Spores are not found. Capsules may sometimes be demonstrated with certain species. Many are actively motile, due to the presence of peritrichate flagella. While the presence of motility is not absolutely constant, *i.e.*, motile forms may be non-motile, it is nevertheless a valuable criterion in the differentiation of some of the species. Thus it is one of the most useful points of distinction between *Bact. typhosum* (motile) and *Bact. flexneri* (non-motile).

These organisms stain readily with the simple dyes. They are decolorized in Gram's method, *i.e.*, they are Gram-negative.

Cultural Characteristics. In all cases growth takes place readily on the ordinary media. These organisms are facultative anaerobes, *i.e.*, growth is most marked under aerobic conditions. Multiplication occurs over a wide range of temperature, 10°–42° C., and the optimum temperature for most species is 37° C. The optimum reaction of the medium is pH 7·4–7·6.

In *fluid media*, such as broth, a well-marked turbidity is usually found in 24 hours. On further incubation a deposit tends to form, but this is readily dispersed on shaking ; pellicle formation is rare.

On *solid media*, such as agar, round, smooth, raised, greyish, semi-opaque colonies are usually produced. On MacConkey's medium the colonies of lactose-fermenting organisms are red, due to the deposition of the neutral red, whereas those of the non-lactose-fermenters are greyish-white. This medium has therefore been of great value in the isolation of the non-lactose-fermenters from material which may contain a large variety of organisms. Characteristic variations are sometimes encountered in the colonial appearance of the different species, especially on their first isolation from the tissues. *Bact. typhosum* may form flat, spreading colonies with an irregular edge, giving the so-called " vine-leaf growth ". *Bact. paratyphosum B*, after incubation at 37° C. for 24 hours and at room-temperature for a further 24 hours, tends to form colonies presenting a sharply differentiated peripheral zone. The colonial characters may thus provide information of some value in the identification of freshly isolated strains.

Resistance. The members of this genus do not exhibit any marked resistance to destructive agents. They are destroyed at 56° C. in 30 minutes. In the case of certain chemical bactericidal agents definite selectivity of action is found. This is particularly marked in the case of dyes, such as brilliant green and malachite green ; coliform and dysentery organisms are most susceptible to their action, *Bact. typhosum* less so, and the members of the Salmonella group are most resistant. The coliforms are also inhibited by tetrathionate, selenites and desoxycholate plus sodium citrate. This variation in the inhibitory activity of these substances has been applied with success to the isolation of the Salmonella organisms from grossly contaminated material.

Stock cultures are easily conserved. A 24-hour growth on agar will remain alive at room-temperature for several months.

Biochemical Activity. Members of this group tend to exhibit marked biochemical activity. The various reactions, such as the fermentation of the different carbohydrates, liquefaction of gelatin and the production of indole, are reasonably constant and they have therefore been employed extensively in the differentiation of the individual species.

In the early studies of this genus it was observed that *Bact.*

coli fermented lactose while *Bact. typhosum* did not. With the isolation of fresh species it was found that lactose occupied a position of great importance in the biological sub-division of this group. Organisms that were normally present in the fæces of man and animals fermented lactose and many other sugars ; on the other hand, with few exceptions, the non-lactose-fermenting members were not normally present in the fæces and were responsible for a number of important gastro-intestinal diseases in man and animals. An arbitrary sub-division of this group into lactose- and non-lactose-fermenters was the natural sequence, and this has been adopted by most workers as a convenient means of simplifying the discussion of this large and unwieldy genus. It is used here, and, as the sub-groups possess many sharply defined characteristics, they are considered separately. It is, however, important to appreciate the essentially close relationship that exists between them, and that irregular results are not uncommonly given by members of this genus.

The Lactose-Fermenting Bacteria

These organisms are widely distributed in nature, but are mainly present in the fæces of man and animals, and are frequently referred to as " coliforms ". The first member to be isolated was *Bact. coli commune*, which was described by Escherich in 1885 ; many others have since been isolated. This group has been subjected to detailed investigations by various workers and several elaborate classifications have been produced. These have been based mainly on extensive series of biochemical tests, but there is little point in examining these classifications closely ; the reactions given by some of the common members of the group are given in Table XII. The coliforms have, however, been conveniently arranged into two main classes :—

(1) Containing those species that are usually found in the intestinal tract, *e.g.*, *Bact. coli* and *Bact. acidi lactici* ; these are loosely termed " fæcal coliforms " ; they all produce indole.

(2) Comprising members that are normally present in the soil and are infrequently present in the intestinal tract, *e.g.*, *Bact. aerogenes* ; these are sometimes termed " non-fæcal coliforms." Some, *e.g.*, *Bact. aerogenes*, do not produce indole, but others, *e.g.*, *Bact. cloacæ*, do.

The differentiation of these two groups is important in the bacteriological examination of water. The presence of the *Bact. coli* group is accepted as an index of fæcal pollution, whereas the presence of the *Bact. aerogenes* group is considered to represent contamination with soil and surface washings from the neighbouring fields. As, however, *Bact. aerogenes* has been isolated from human fæces, the value of this distinction is open to question. The important differential points between the two groups include the production of indole, the Voges-Proskauer (V.P.) test, the methyl-red (M.R.) test and the fermentation of various carbohydrates (see Table XII.). Other criteria employed in the identification of these organisms are the gas-ratio, *i.e.*, relation of H_2 to CO_2 in gas formed by fermentation of sugars, growth at 44° C., and growth on uric acid and citrate media. These points are considered later (Chapter XXXV.).

The fermentation of lactose is the important criterion of this sub-group, although certain organisms, possessing the generic characters but either not fermenting lactose or fermenting this sugar atypically, are accepted as members. These closely resemble the ordinary coliforms in other respects; they form indole and are members of the normal intestinal flora. They are frequently referred to as the "paracolon" bacteria. Some of these organisms are late-lactose-fermenters, some ferment lactose with the production of acid only and others do not ferment it. The members of this last group are considered by some workers to be a connecting link between the coliforms and the Salmonella

TABLE XII

Biochemical Reactions of the Common Coliforms

	Lactose	Sucrose	Dulcite	Glucose	Gelatin	Indole	V.P. Test	M.R. Test	Motility	Milk
Bact. coli commune .	+	—	+	+	—	+	—	+	+	A+C
Bact. coli communior .	+	+	+	+	—	+	—	+	+	A+C
Bact. acidi lactici .	+	—	—	+	—	+	—	+	—	A+C
Bact. aerogenes . .	+	+	—	+	—	—	+	—	—	A+C
Bact. cloacæ . .	+	+	—	+	+	+	+	—	+	A+C

KEY + = acid and gas in the case of the sugars and a positive result in the other tests.
— = negative result.
A+C = acid and clot.

group; they have, however, no definite pathogenic *rôle* in the intestinal tract. The paracolon group can usually be differentiated from the Salmonella organisms by their lack of motility and their tendency to produce indole.

Serology. Serological tests have not been employed to any great extent in the study of the lactose-fermenters. The available results indicate that a complex antigenic structure is present in most species; there is no agglutination with the Salmonella or dysentery groups. These tests have no practical application in the identification of the coliforms.

Pathogenicity. The lactose-fermenting bacteria are non-pathogenic when present in the intact intestinal tract, but if they pass from the intestinal tract to other tissues, pyogenic lesions may develop. Pyelitis, cystitis, empyema, pelvic abscess and cholecystitis may be caused by these organisms, while one of the most serious coliform infections is acute peritonitis following perforation of the intestinal wall.

The coliform lesions of the urinary tract as a rule respond well to vaccine therapy; sulphonamide preparations also give good results.

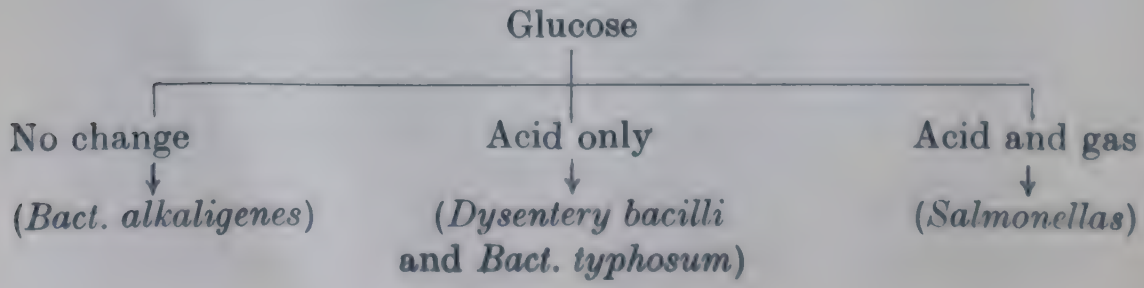
In addition to the coliform organisms there are two other species of this genus that ferment lactose; these are *Bact. sonnei* which is responsible for many cases of dysentery in this country, and *Bact. dispar* which is, however, a doubtful pathogen. In both cases acid, but not gas, is produced usually after several days' incubation and both are non-motile. These organisms are considered later with the dysentery group, to which they are more closely related.

Non-Lactose-Fermenting Bacteria

This group includes a large number of pathogens and has been conveniently sub-divided by the action of the various members

TABLE XIII

Sub-division of the Non-lactose-fermenters by Glucose Fermentation



on glucose. By this means three main sub-groups are formed (Table XIII.).

The first sub-group contains one species, *Bact. alkaligenes*, which does not ferment any sugars. Its exact taxonomic position is uncertain; some workers have placed it in the Brucella genus, but as it possesses some of the properties of the group, it is usually included as a member of the Bacterium genus. *Bact. alkaligenes* is normally present in the fæces; it has also been isolated from the blood and fæces of cases of enteritis, for which it has been incriminated as the ætiological agent. This pathogenic *rôle* is, however, open to doubt.

The sub-group fermenting glucose with the production of acid only includes the dysentery bacilli, the typhoid bacillus and *Bact. gallinarum*, a pathogen in fowls. The last two members are closely related to, and are usually discussed with, the Salmonella group.

Members of the third sub-group are motile and ferment glucose with the production of acid and gas. They include those bacteria causing enteric fever and gastro-enteritis in man. These have serological relationships with *Bact. typhosum* and *Bact. gallinarum*, with which they have recently been placed in a separate genus "Salmonella." All these organisms are, however, considered here as a sub-group and not as a distinct genus.

The Salmonella Group

In an early report of the Salmonella Sub-Committee of the International Society of Microbiology forty-four members were included in this group, but at the moment more than 140 types have been identified; many of these are rarely, if ever, found in this country. Important in medicine are those causing enteric fever and gastro-enteritis in man. Responsible for the former condition are *Bact. typhosum*, *Bact. paratyphosum A*, *B and C;* certain workers have given the name *Bact. paratyphosum C* to various organisms, but two types only are now recognized as ætiological agents of enteric fever: (1) The Western type or *Bact. choleræ-suis* (*suipestifer*) (monophasic), which is present in Western Europe, and (2) the Eastern or Hirschfeld's type (*Bact. paratyphosum C*), which is common in Eastern Europe and Asia. Many organisms are responsible for gastro-enteritis; those most frequently encountered in this country are *Bact. typhi-murium* (*aertrycke*) and *Bact. enteritidis* (Gaertner's bacillus).

Bact. morgani, which some consider to be one of the causative agents of summer diarrhœa, is not included in this group.

Members of the Salmonella group are all motile and exhibit saccharolytic activities which tend to be constant. Fermentation tests are therefore useful in establishing the identity of the various members ; sugars particularly useful in differentiation are xylose, inosite, arabinose and litmus-milk (see Table XIV.).

TABLE XIV

Biochemical Reactions of the Salmonella Group

Species	Motility	Lactose	Sucrose	Maltose	Mannite	Glucose	Dulcite	Xylose	Inosite	Arabinose	Litmus-milk	Indole	Gelatin
Bact. typhosum .	+	–	–	A	A	A	–	A	–	–	A	–	–
Bact. paratyphosum A	+	–	–	A+G	A+G	A+G	(A+G)	–	–	A+G	A	–	–
Bact. paratyphosum B	+	–	–	A+G	A+G	A+G	A+G	A+G	(A+G)	A+G	A⟶Alk	–	–
Bact. typhi-murium .	+	–	–	A+G	A+G	A+G	A+G	A+G	A+G	A+G	A⟶Alk	–	–
Bact. enteritidis . .	+	–	–	A+G	A+G	A+G	A+G	A+G	–	A+G	A⟶Alk	–	–
Bact. suipestifer . .	+	–	–	A+G	A+G	A+G	A+G	A+G	–	–	A⟶Alk	–	–

KEY. + = positive. – = negative. A = acid. A⟶Alk = acid becoming alkaline. A+G = acid and gas. (A+G) = sometimes acid and gas.

Serology. The biochemical tests provide useful but not conclusive evidence of identification, which is definitely established by serological methods usually in the form of agglutination tests. In fact, it is mainly in the study of these organisms that our knowledge of the mechanism of agglutination has been obtained. The various factors involved in the agglutination reaction have already been considered in Chapter IX. In view of the importance of this test in routine practice it appears advisable to mention briefly the two main factors responsible for the difficulties that arise in the interpretation of the results given by this group :—

(1) The relationship between bacterial variation and antigenic structure is implicated. Before the possible antigenic changes can be appreciated the normal antigenic structure of these organisms must be understood.

The S or normal, smooth, motile form contains O (somatic) and H (flagellar) components. Both of these components are variable and several arrangements may consequently be found.

(*a*) Diphasic changes in the H antigen may be encountered. When an organism manifests this change there are two possibilities :—

(i) S may contain O + H (phase 1), *i.e.*, type phase, in which the organism exhibits a high degree of specificity.

(ii) S may contain O + H (phase 2), *i.e.*, usually, but not necessarily, group phase.

(*b*) Another possibility is the change of the motile to the non-motile form, as represented by the variation :

O + H → O.

(*c*) In the variation, smooth to rough (S → R), the following antigenic changes may occur :—

(i) O + H → Ø + H (motile, rough form).
(ii) O + H → Ø (non-motile, rough form).
(iii) O → Ø (non-motile, rough form).

(2) The presence of common antigenic components in different species is also a source of confusion. It has been stated that, in many cases, the O and H components are not simple antigens, but that each may contain several distinct agglutinogens. The presence of any one of these separate fractions in different bacterial species may result in cross agglutination. The antigenic structure of the common Salmonellas as indicated descriptively by symbols illustrates this point (see Table XV.). A practical sub-division of the Salmonellas has been made on the basis of the somatic antigens.

TABLE XV

Antigenic Structure of the Common Salmonellas

(Kauffmann-White Scheme)

Group	Type	Somatic or "O" antigens	Flagellar or "H" antigens	
			Phase 1 (type)	Phase 2 (group or type)
A	*Bact. paratyphosum A* .	(I), II, XII	a.	—
B	*Bact. paratyphosum B* .	(I), IV, (V), XII	b.	1, 2...
	Bact. typhi-murium (ærtrycke).	(I), IV, (V), XII	i.	1, 2, 3...
	Bact. stanley .	IV, V, XII	d.	1, 2...
C	*Bact. paratyphosum C* .	VI, VII	c.	1, 5...
D	*Bact. typhosum* . .	IX, XII	d	—
	Bact. enteritidis . .	(I), IX, XII	g,m...	—

KEY. ( ) = antigen may be absent.

The sharing of antigenic components is a common feature, *e.g.*, *Bact. typhosum* and *Bact. enteritidis* share common O factors, while *Bact. paratyphosum B* and *Bact. typhi-murium* (*aertrycke*) not only have O factors in common, but they also possess two identical group H components.

In order to obtain satisfactory results in the practical application of agglutination, all possible variations and irregularities must be anticipated. In the serological diagnosis of Salmonella infections the patient's serum must be tested against various O and H bacterial suspensions, so that all possible antigenic factors are included. The O suspensions are obtained by treating a suspension of an agar culture with absolute alcohol. H suspensions are formolized suspensions ; these are not true H suspensions as some O factor is present, but this tends to be inhibited by treatment with the formalin. In this country the types usually encountered are *Bact. typhosum*, *paratyphosum B*, *typhi-murium* and *enteritidis ; Bact. paratyphosum A* is extremely uncommon, but as it may be responsible for infections in individuals who have travelled abroad, the H suspension is included in the routine series of suspensions. Therefore in routine work at least six suspensions, plus one of *Br. abortus* (*q.v.*), should be employed (see Table XVI.).

TABLE XVI

Suspensions Employed in Routine Practice

Suspension	Types giving a reaction
(1) *Bact. typhosum* H . .	typhosum and stanley
(2) *Bact. typhosum* O . .	typhosum, enteritidis (Group D) and paratyphosum B.
(3) *Bact. paratyphosum* A " H " (specific)	paratyphosum A
(4) *Bact. paratyphosum* B " H " (specific)	paratyphosum B
(5) *Bact. paratyphosum* B " O "	paratyphosum B and some of the food-poisoning group (Group B).
(6) Group " H " . . .	paratyphosum B and the food-poisoning members in the group phase.

By the use of such a scheme most members can be detected; in the event of marked reaction only with suspension six, further examination with suspensions of individual members is necessary.

Similarly in the identification of a bacterium suspected of being a member of this group, it is necessary to test it first against those sera indicated by the fermentation tests. These sera are usually prepared by intravenous injection of the bacterial suspensions in the rabbit. In some cases either sera prepared with the organism in the type phase or agglutinin-absorption tests may finally be necessary to establish the identity of the organism.

It may occasionally happen that irregular results are obtained suggesting the discovery of a new or rare species. However, before such a claim is made it is recommended that there should be found either distinctive cultural characters or differences in the O antigen and/or the specific phase of the H antigen from those already described. In all cases of doubt the organism should be sent to a Salmonella Reference Laboratory for identification.

Pathogenicity. The members of the Salmonella group are essentially pathogenic to man and animals. In man two important clinical conditions may be produced: (*a*) enteric fever caused mainly by the typhoid and paratyphoid bacilli, and (*b*) gastro-enteritis resulting from infection with organisms associated with food-poisoning, *e.g.*, *Bact. typhi-murium* (*aertrycke*) and *enteritidis*.

In experimental animals a condition analogous to the human infections cannot be produced. Intravenous or intraperitoneal inoculation is followed by death in 18–72 hours, and on autopsy a generalized infection is generally found.

Enteric Fever

Enteric fever is a disease of great antiquity and world-wide distribution. In Great Britain it was prevalent to an appreciable extent until the end of the last century, when marked advances were made in the general hygienic arrangements.

Although many striking epidemiological studies had been made previously, knowledge was greatly advanced by the description of the typhoid bacillus by Eberth in 1880 and its isolation in 1884 by Gaffky. The condition subsequently attracted the attention of many bacteriologists, and it was found that *Bact. paratyphosum A* and *Bact. paratyphosum B* were also responsible for enteric fever (Schottmüller, 1900). Infections with the paratyphoid bacilli are, however, generally less severe and less fatal than those with the typhoid bacillus. *Bact. paratyphoid B* infections are much more frequent in temperate climates than infections with *Bact. paratyphosum A*, the latter organism being mainly encountered in tropical regions.

Pathogenesis. Enteric fever occurs spontaneously only in man. Subcutaneous, intraperitoneal or intravenous inoculation of laboratory animals is followed by septicæmia and death; this type of infection is, however, not strictly analogous to that found in man.

Infections in man originate directly or indirectly from the excreta of infected humans, either from actual cases or carriers, the latter being more dangerous. The portal of entry of the causative bacteria is the mouth, and the most frequent vehicles of infection are contaminated food or drinking-water. Contamination may be due to fæcal pollution of the water supply or may be conveyed by the hands or flies. Shell-fish, such as oysters, may also be responsible for the transmission of infection, as they sometimes become contaminated with sewage during growth on the river-bed. Recent examples of enteric fever in this country are the Bournemouth outbreak in 1936 in which 700 cases occurred and the vehicle of infection was milk, and the Croydon outbreak in 1937 for which the water supply was responsible. In both cases carriers were the original source of the infection.

The course of events after entrance into the alimentary tract has not been definitely settled, but the old view that the bacilli multiplied in the intestines and then passed into the blood-stream has been generally discarded. Clinical and bacteriological observations indicate that the bacteria proliferate primarily in the spleen, liver and mesenteric glands. The incubation period is long, about 14 days, and, during this time, the bacteria pass from the alimentary tract probably *viâ* the intestinal lymphatics to these tissues, where multiplication takes place. At the end of this period the bacteria pass into the blood-stream and produce a bacteræmia and the associated symptoms. After this, bacteria appear in the intestines either by way of the gall-bladder or from Peyer's patches. The bacteria invade and multiply in Peyer's patches, which break down and slough; this process may be responsible for severe hæmorrhage or perforation of the gut, both of which are serious complications. The organisms are usually present in the gall-bladder, which appears to be infected from the blood-stream. Cholecystitis not infrequently develops, and gall-stone formation is a relatively common sequel ; this favours the persistence of the carrier state. It is interesting to note that living typhoid bacilli have been isolated from the centre of gall-stones. The bacteria are also frequently excreted by the kidneys in the urine. When this excretion persists and the individual becomes a urinary carrier, there is usually an associated lesion in the urinary tract, frequently in the kidney or kidney-pelvis.

Carriers. The fæcal and urinary excretion of the causative organisms frequently persists throughout convalescence, during which it gradually becomes less marked until it finally ceases. These cases are transitory or temporary carriers. In a few instances, 2–4 per cent., the excretion, fæcal and/or urinary, persists, giving rise to the chronic or permanent carrier. These cases seldom clear spontaneously. The discharge may be intermittent, but nevertheless the uncontrolled carrier is always a potential source of danger to the community. These unfortunate individuals are almost invariably responsible for the endemic outbreaks occurring in this country. Women are more frequently fæcal carriers than males ; this is probably due to the greater incidence of cholecystitis and gall-stones in females than in males. Urinary carriers are less common than fæcal carriers, but they constitute a more serious danger to the community because of the greater facilities for disseminating the organisms.

Diagnosis. The pathogenesis of the condition indicates that different specimens should be examined according to the duration of the infection. Materials giving valuable results are (1) blood, (2) fæces, (3) urine and (4) serum.

(1) **Blood.** The most useful results in the early stages are obtained from blood culture. In the first week the bacterium can be isolated in 90–95 per cent. of cases, in the second week the percentage of positives is less and after the fourth week the blood is rarely positive. Some 5–10 c.c. of blood are collected and transferred to 50–100 c.c. of broth or fluid MacConkey medium. After incubation at 37° C. for 24 hours subcultures are made on to a solid medium, such as MacConkey's or Russell's double sugar (*q.v.*).

(2) **Fæces.** During the early stages the fæces are frequently free from the infecting organism. After the first few days some 50 per cent. of cases are positive, and by the third week this figure is increased to 70–80 per cent. After this there is a sharp decline. The isolation of the bacteria from the fæces is some-

times difficult. Spread cultures should be made on to selective media, such as desoxycholate-citrate, Wilson and Blair, and into tetrathionate or selenite broth ; if the fæces are solid, they must first be suspended in saline or peptone water. After incubation at 37° C. for 24 hours, the cultures are examined. In the case of the solid medium, discrete non-lactose-fermenting colonies are picked off and subcultures prepared in peptone water or on Russell's double sugar medium ; biochemical and serological tests are ultimately employed for identification. Subcultures from the fluid media are made on to MacConkey's medium, from which non-lactose-fermenters are picked off and examined as above.

(3) **Urine.** Urine should be collected under aseptic conditions and examined in a similar manner to the fæces. Typhoid bacilli are frequently isolated in the second, third or fourth week and may be present in large numbers. The urine may be centrifuged to concentrate the organisms in the deposit, which is used for the examination.

(4) **Serum.** The application of the agglutination test in the diagnosis of enteric fever has evolved considerably since the method was originally introduced by Widal. The use of a simple suspension of *Bact. typhosum* yields information of an inconclusive nature and, as previously indicated, other bacterial suspensions are necessary to ensure a satisfactory result. The routine test of the present time contains at least six suspensions, which include the H and O factors of the three members usually responsible for enteric fever, and a group suspension in order to pick out any other member likely to be involved. These suspensions are standardized and issued by the Medical Research Council, and their general use tends to obviate irregularities due to unsatisfactory suspensions. As a further refinement each suspension may have a " reduced " titre, which is an index of agglutinability and is obtained by testing each suspension against a fixed standard. The agglutination titre of the serum is reduced by the fraction indicated for each suspension, and it is considered that the reduced titres obtained by the use of all standard suspensions of the same organism are strictly comparable.

Agglutinins appear usually towards the end of the first week of the disease and the titre increases until the fourth week, after which there is a fall, marked at first, but slow later. Agglutinins may be demonstrated some time after recovery. In infections with the typhoid bacillus, the O agglutinin response is frequently more marked than the H. The titre varies considerably, but one over 1/100 with either the H or O suspension is practically diagnostic in an uninoculated individual. Agglutinins may be present in the serum of normal individuals, but these seldom give a titre above 1/25 and the O agglutinins appear to be more common than H agglutinins.

In the case of *Bact. paratyphosum B* infections, H agglutinins are more marked and high titres are usually recorded. The O response is not infrequently poor.

In both cases cross-agglutination with the O suspensions of *Bact. typhosum* and *Bact. paratyphosum B* often takes place. While there is no doubt about this cross agglutination, the presence of a common O antigen was not indicated in any of the earlier published schemes indicating the antigenic structure of this group.

Agglutinins are also produced by prophylactic inoculations of the T.A.B. vaccines. This response can usually be differentiated from that manifested in the actual infection. Following inoculations of the T.A.B. vaccines, H agglutinins are elicited more readily than O agglutinins, which can frequently not be detected unless the examination is made within a short time after the injections. The titres of the H agglutinins may, however, be high, particularly if the inoculations have been recent, titres as high as 1/2560 have been observed ; H agglutinins for the typhoid and paratyphoid bacilli are generally all present to a similar titre. This is not usual in infection.

It is important to note that virulent strains of *Bact. typhosum* may be inagglutinable on account of the antigenic component, termed " Vi " (p. 125). The Vi antigen is labile and agglutination tests should be carried out with bacterial suspensions heated at 100° C.

Phage Typing. Bacteriophages acting on the Vi antigen of *Bact. typhosum* have recently been demonstrated by Craigie and his colleagues. Four distinct phages have been identified ; one, type II, exhibits a high degree of strain selectivity and can be " conditioned " to particular strains by appropriate changes of the substrate. These conditioned phages are stable and are used to type strains of *Bact. typhosum* ; the following main types have been identified—A, B, C, D, E, F, G, H, J, L, M. Phage typing requires considerable experience and it is used mainly for epidemiological purposes, *e.g.*, for tracing the source of infection and relationship between cases.

Prophylaxis. Prophylaxis has played an important part in the elimination of enteric fever from civilized communities. General and specific measures have been adopted.

The general measures adopted in the prophylaxis of enteric fever have been numerous. Improvement in sanitary arrangements, particularly with regard to sewage disposal, has been an important step, while the control of possible vehicles of infection, such as drinking-water, milk, ice-cream, shell-fish and flies, has been another valuable contribution. Another important factor is the control of carriers, though unfortunately their complete elimination is impossible. When endemic outbreaks occur carriers are almost invariably responsible, and rigorous attempts should be made to locate them ; cultural and serological examinations have given the most satisfactory results in identifying carriers. It has been stated that some 2–4 per cent. of all cases become permanent carriers, either fæcal or urinary, and that female carriers greatly outnumber males. As women are more frequently concerned with the preparation of food, the danger is apparent. As an illustration, brief mention may be made of the celebrated " Typhoid Mary " case. " Typhoid Mary " was a cook in America who, in 1901, developed enteric fever. She subsequently was employed in different houses and in many was responsible for several cases of enteric fever ; in 5 years she was known to have caused 26 cases. She was then placed under the control of the Health Department of New York, but in 1914 she escaped observation. In 1915 an outbreak involving 25 cases occurred in a hospital ; it was discovered that the cook had disappeared and on location she proved to be " Typhoid Mary." She was then kept under stricter control and has recently died. It was thought that she was also responsible for other outbreaks, including one involving over 1,300 individuals.

The detection of carriers would doubtless be facilitated by the adequate bacteriological control of the cases at the time of the infection. Cases should be kept in quarantine until at least three successive bacteriological examinations of the urine and fæces, carried out at weekly or fortnightly intervals, have given negative results. It is claimed that practically all carriers of *Bact. typhosum* possess Vi agglutinins in a titre of 1 in 10 or over. Agglutination tests must be carried out at 37° C., in 3 × ½ in. test-tubes, using a specially prepared bacterial suspension, *i.e.*, one containing Vi antigen.

The control and treatment of the chronic carrier is unfortunately an extremely difficult problem. The only satisfactory measure is by surgical operation ; cholecystectomy in the case of the fæcal carrier and the operative treatment of gross lesions in the case of urinary carriers. Other measures, such as drugs and vaccines, have proved useless. In all cases personal instruction of the potential danger and the precautionary measures to be adopted must be given by the medical officer.

Specific immunization by means of vaccines has yielded very satisfactory results. Since the introduction by Wright of the T.A.B. vaccine the incidence of enteric fever in exposed individuals has decreased considerably. In ordinary communities the necessity for such a prophylactic procedure does not arise, but where the risk of exposure is increased, such as in military operations in foreign countries or by visits to tropical regions, vaccines have proved particularly useful. Statistics indicating the value of T.A.B. inoculations in the Army during the two Great Wars are readily available ; there are no adequate control groups as inoculation is compulsory. The following figures, collected by the Anti-typhoid Committee (1913), indicate the striking results of their use (Table XVII.).

Table XVII

	Number	Number Attacked
Inoculated . . .	10,378	56
Not inoculated . .	8,936	272

The usual vaccine contains 1,000 million *Bact. typhosum* and 500 million each of *Bact. paratyphosum A* and *Bact. paratyphosum B*. The initial dose is 0·5 c.c. given subcutaneously, followed by a second dose in 7–10 days of 1 c.c. Smooth virulent strains must be used in the preparation of the vaccines, and in ? order to preserve the Vi antigen it is now recommended that alcohol-killed and alcohol-preserved vaccines should be used. Mouse protection tests indicate that the alcoholized vaccine is more efficient than the old heat-killed and phenol-preserved type. A further injection should be given at the end of 18–24 months if the exposure to risk is continued.

Therapy. Specific therapeutic measures have been generally unsatisfactory. The recent claims of success with " Vi " antiserum by Felix and his colleagues, however, appear to be very promising. Bacteriophage has been recommended, but the results have been disappointing. Sulphonamides and penicillin have so far not been successful.

Bacterial Food-poisoning

Food-poisoning may result from the ingestion of either chemical poisons or bacteria. We are concerned here with the bacterial type. At one time many considered that food-poisoning was due to the action of ptomaines, and the clinical condition was usually termed " ptomaine poisoning." On investigation, this view was found to be erroneous and the use of the term " ptomaine poisoning " is to be condemned: Ptomaines are products of protein disintegration, and experimental evidence obtained in laboratory animals indicates that they are relatively non-toxic and that they are not concerned in cases of food-poisoning.

While the members of the Salmonella group are probably the main bacterial agents causing food-poisoning, they are not solely responsible for this condition ; *Bact. flexneri*, *Bact. sonnei*, *Staphylococcus*, *Proteus*, *Bact. morgani* and *Cl. botulinum* (*q.v.*) have all been incriminated in various outbreaks.

Various members of the Salmonella group have been isolated from outbreaks of food-poisoning ; most frequent in this country are *Bact. typhi-murium* and *Bact. enteritidis ;* others have been isolated at different times and have frequently been called after the place where they were isolated : *Bact. derby*, *stanley*, *newport*, *dublin*, *thompson*, *choleræ suis* and *oranienburg*.

Bact. enteritidis was first isolated from cases of food-poisoning in Germany by Gaertner in 1888 ; 57 cases of acute gastro-enteritis occurred in individuals who had eaten meat taken from a condemned cow. The organism was obtained both from the cow and a fatal case.

Bact. typhi-murium (*aertrycke*) was first described by De Nobele, who in 1898 isolated it during an outbreak in Belgium from both the patients and the meat consumed by them.

The clinical picture of bacterial food-poisoning is that of an acute gastro-enteritis. The time of onset varies, but is usually 4–36 hours after ingestion of the infected food, and is characterized by nausea, abdominal discomfort, vomiting, headache and later diarrhœa. The symptoms are frequently associated with a moderate degree of pyrexia. In most cases these symptoms subside in a few days and the patients are completely recovered in 6–8 days. The mortality rate is low, approximately 1 per cent. In fatal cases the infection is generalized and the causative organism can be isolated from the intestinal tract, blood, liver spleen, lymph glands, etc.

The type of food involved in food-poisoning is usually meat, milk, eggs and fish. The meat is frequently some preparation, such as pies, pastes, sausages and brawns, which is insufficiently cooked and has been subjected to much handling ; contamination of these foods results either from disease of the animal or from an outside source during preparation of the food. The former cause is probably less frequent than the latter, and is responsible for *Bact. enteritidis* rather than *Bact. typhi-murium* infections. *Bact. enteritidis* is an important pathogen in cattle, and the tissues of an infected animal might be contaminated either as a result of a generalized infection or by contamination with fæces at the time of slaughter. Eggs from infected ducks have also been responsible for several outbreaks, while many types of Salmonella have been isolated from dried egg powders.

Food may also become contaminated from an outside source, usually by animal agents. Human carriers of Salmonella organisms are rare, but such individuals have ample opportunity of introducing these organisms into any food that they might be preparing. Several outbreaks in the troops during the last war were traced to carriers in the cook-house.

Salmonella infections are very common in rodents, in which epidemics are frequent. Rats and particularly mice have been incriminated in a number of outbreaks as the original source of infection. Prepared foods are contaminated by the excreta of these animals and, as the foods are usually excellent media, the bacteria multiply readily. This is considered to be the method of contamination in many outbreaks of food-poisoning due to *Bact. typhi-murium.*

Members of the Salmonella group, particularly *Bact. enteritidis*, have been employed in various preparations, such as Liverpool Virus and Rattin, for exterminating rats. The idea is to introduce the infecting agent into the rat population and produce epidemics. Their use has, however, not been particularly successful. Acute and fatal attacks do not invariably result; the condition may become chronic and the carrier rate of the organism in the local rodent population would consequently be greatly increased. Also when fatal the bodies may remain hidden and putrefy with unpleasant results.

Food-poisoning in this country is most frequent during the summer months. Two factors are largely responsible for this; the temperature is, at that time, most suitable for bacterial growth and also prepared foods, *e.g.*, potted meats, sausages, etc., are eaten in greater quantities than during the winter months.

Diagnosis. Whenever the clinical signs indicate food-poisoning, examinations should be carried out, if possible, on the following materials :—

(1) Vomit.
(2) Fæces.
(3) Blood for organisms and agglutinins.
(4) Suspected food.
(5) Blood and fæces of suspected carriers.

In many cases the vomit is discarded and is therefore not available for examination, but, if possible, cultures should be made on suitable selective media, *e.g.*, desoxycholate-citrate, following the same procedure as in the examination of fæces for members of this group. Except in fatal cases, blood cultures are usually negative. Agglutinins are not present in the early stages, but their appearance some 10–14 days after the onset of the illness might provide useful diagnostic evidence, particularly if the cultural tests are negative.

The nature of the suspected food is traced by the history and extent of the outbreak. The morbidity rate is high, and all people partaking of the food usually manifest some sign of infection. In many cases the food is thrown away, but wherever possible cultures should be prepared in a selective broth medium. In practically all instances the food does not present any gross evidence of contamination with the food-poisoning organisms; there is usually no alteration in smell, taste or appearance.

All suspected human carriers should be subjected to serological and cultural examinations. When the rooms where the incriminated food has been stored are overrun with rodents, these should be trapped and examined for the presence in the intestines of members of the Salmonella group.

In quite a number of cases the bacteriological examinations have been negative. In many instances this has probably been due to the unsatisfactory nature of the bacteriological investigations, but in a few cases this objection does not appear to be justified. The ætiology of these cases is uncertain; it has been suggested that the Salmonella organisms are able to produce a thermo-stable toxin, capable of producing symptoms of gastro-enteritis. The symptoms of intoxication appear early, after ½–6 hours, and soon subside. Confirmation of this theory is, however, necessary before it can be finally accepted.

Prophylaxis. The preventive measures likely to reduce the incidence of food-poisoning are :—

(1) Adequate meat inspection at the slaughter-house.

(2) Extermination or exclusion of rodents from places where food prepared for human consumption is stored.

(3) Adequate cooking of prepared foods and dried egg powders.

(4) Conservation of the food at low temperatures, *e.g.*, refrigeration.

(5) Control of Salmonella carriers.

The Dysentery Group

Members of the dysentery group of the *Bacterium* genus are non-motile, ferment various carbohydrates with the production of acid, but not gas, and are serologically distinct from the members of the Salmonella group.

Dysentery is usually considered in two main classes, (1) amœbic and (2) bacillary. The relationship of amœbæ to dysentery was described by Loesch in 1875, but the bacillary form was not recognized until some time later. The first recognized species (*Bact. shigæ*) was described in 1898 by Shiga, who isolated it from a number of cases of dysentery during an outbreak in Japan and considered it to be the ætiological agent. Two years later, 1900, Kruse obtained a similar organism from dysentery cases in Germany. About the same time Flexner and Strong isolated organisms, resembling but not identical with that described by Shiga, from cases in the Philippine Islands. Subsequently other types were isolated in various parts of the world, but although these resembled the dysentery bacilli in some respects, the strict relationship of these organisms to the disease process was not always established. It is therefore not surprising that the classification of this group was for many years uncertain and indefinite.

The first systematic attempt at sub-division of this group was made by Lentz in 1902. Two sub-groups were made, based on the capacity to ferment mannite; the Shiga group did not ferment mannite, while the group described by Flexner and Strong fermented this carbohydrate. Hiss (1904–5) amplified this scheme and distinguished four groups on fermentation and serological results ; in this scheme the mannite-fermenters were divided into three sub-groups. Although this classification was not generally accepted, it was recognized that while the Shiga group was homogeneous the Flexner group contained a very heterogeneous collection of organisms.

During the Great War many cases of dysentery were examined and further types were described, *e.g.*, Sonne, Alkalescens of Andrewes, and Schmitz. Much work has been carried out in attempts to produce a satisfactory scheme of classification. The results are not as yet complete, but five main types are now generally recognized : Shiga, Flexner, Sonne, Schmitz and Boyd. The pathogenicity of *Bact. alkalescens* is very doubtful. A further organism, the Newcastle bacillus, was isolated from an outbreak of dysentery at Newcastle in 1929 ; this differs from other members of this group in being motile on first isolation and producing acid and gas in certain sugars, *e.g.*, glucose, but it belongs serologically to the Flexner group and is now designated *Bact. dysenteriæ* (Flexner VI).

Biochemical Activity. The biochemical activities of most members of this group are sufficiently constant to enable them to be employed as a basis of various classifications. They are essentially non-lactose-fermenters, but, as stated previously.

TABLE XVIII

Biochemical Reactions of the Dysentery Group

Species	Motility	Lactose	Glucose	Mannite	Dulcite	Litmus-milk	Indole
Bact. shigæ . . .	−	−	+	−	−	Sl.A	−
Bact. flexneri . . .	−	−	+	+	−	Sl.A	+−
Bact. sonnei . . .	−	+−	+	+	−	A+C	−
Schmitz's bacillus . .	−	−	+	−	−	Sl.A	+

Bact. sonnei, a late-lactose-fermenter, is included because it is ætiologically responsible for cases of dysentery.

The fermentation of mannite provides a useful means of subdivision ; *Bact. shigæ* and Schmitz's bacillus are non-mannite-fermenters, while other members are mannite-fermenters (Table XVIII.). The action on other sugars is of little use in the differentiation of the various members of this group.

In the case of proteolytic activity, indole production is of greatest practical value. Indole is produced by Schmitz's bacillus, but not by *Bact. shigæ ;* it thus provides a means of differentiating the non-mannite-fermenters. It is also formed by many strains of *Bact. flexneri*. The various biochemical reactions, important in the identification of the members of this group, are given in Table XVIII.

Serology. Agglutination is the serological test usually employed in the examination of these organisms, but in certain cases the results have been somewhat irregular. The behaviour of the non-mannite-fermenters has, however, been consistent and the strains of *Bact. shigæ* appear to be homogeneous. They react with serum prepared against one strain and exhibit little if any reaction with sera prepared against *Bact. flexneri* or Schmitz's bacillus.

The other group, *i.e.*, the mannite-fermenters, has presented more difficulty. *Bact. flexneri* has a complex antigenic structure, at least four specific antigenic components having been described. These components appear to vary in the various strains, and, according to the distribution of these antigenic factors, Andrewes and Inman (1919) classified strains of *Bact. flexneri* into five main serological sub-groups : V, W, X, Y and Z. This subdivision was founded largely on an extensive series of cross-agglutination tests.

Boyd recently reinvestigated the Flexner group, using many strains isolated mainly in India. He found that V, W, and Z types had separate, distinct specific antigens and that all shared common group antigens. Cross-agglutination between these types was due to the common, group or non-specific antigens of which there were probably at least six different components. During artificial cultivation the specific antigens were lost and the organisms acquired solely group characteristics. The change from the specific phase to the group phase tended to occur slowly but with some strains it took place rapidly.

Boyd found that (1) the Y type contained solely group antigens and, as no such type has been isolated directly from cases of dysentery, he considered the Flexner Y type to be a laboratory product resulting from the loss of the specific antigens by the other types during artificial cultivation, and (2) X type was a degraded form of the Z type. It is therefore important to note that in order to obtain reliable typing of members of the Flexner group recently isolated strains should be used both in the preparation of the antisera and also for the agglutination tests.

Boyd also described other types of dysentery bacilli some of which had definite affinities to the Flexner group but which had special specific antigens. These have been designated types 103, 88 and P 119; type 88 appears to be serologically identical with the Newcastle bacillus. The following classification of the mannite-fermenting dysentery bacilli (*Bact. dysenteriæ*) is now recommended :—

New	Old
Flexner I	Flexner V
Flexner II	Flexner W
Flexner III	Flexner Z
Flexner IV	Flexner (Boyd) type 103
Flexner V	Flexner (Boyd) type P 119
Flexner VI	Flexner (Boyd) 88 ; Newcastle
Boyd I	Flexner (Boyd) 170
Boyd II	Flexner (Boyd) P 288
Boyd III	Flexner (Boyd) D 1

Bact. sonnei is antigenically distinct from *Bact. flexneri*. Agglutination tests with *Bact. sonnei* may be complicated by the fact that this organism may become rough on artificial cultivation. It should therefore be tested as soon after isolation as possible.

Toxicity. *Bact. shigæ* differs from the other members of the dysentery group in that it produces an active toxin, which possesses many of the properties of the so-called "exotoxins". It is obtained by filtering either suspensions of 24–36 hour cultures on agar or broth cultures after 5–7 days' incubation at 37° C. It is destroyed by heat at 75°–80° C. in 1 hour. The intravenous injection of small doses into rabbits is followed by collapse, diarrhœa and paralysis of the limbs. The specific antitoxin, produced by the inoculation of the horse, has proved a useful therapeutic agent.

Bact. flexneri and *Bact. sonnei* form a so-called "endotoxin". Death is produced on the injection of rabbits, guinea-pigs and mice with large doses of a killed culture.

Pathogenicity. While dysentery is considered to be essentially a disease of man, the author has encountered the spontaneous disease in *Rhesus* monkeys during work on poliomyelitis and vaccinia; the course was comparable to that seen in the severe human form. The monkeys were carriers of the dysentery organisms. The experimental condition is, however, quite different from the spontaneous infection. The subcutaneous, intravenous or intraperitoneal injection of laboratory animals with the various organisms results in death which is due to intoxication; the condition is thus quite distinct from dysentery in man. Attempts to produce experimental dysentery by feeding the organisms by the mouth have been fruitless.

In bacillary dysentery the organisms are introduced by the mouth usually by means of contaminated food or water. After a short incubation of 12 hours to several days, abdominal pain and intense diarrhœa appear. These symptoms increase in severity; colic, tenesmus and the appearance of blood and mucus in the fæces are common features. In some cases, particularly in *Bact. shigæ* infections, nervous symptoms, such as muscular twitchings and prostration, are seen. The condition remains localized to the large intestine, and the organisms are rarely isolated from the blood-stream and never from the urine.

In some cases the lower part of the ileum may be involved. In the initial stages there is a catarrhal inflammation and congestion of the mucosa, which subsequently undergoes necrosis, sloughing and ulceration. *Bact. shigæ* infections tend to be more serious than *Bact. flexneri* or *sonnei* infections; the mortality rate is greater, in some cases up to 20 per cent., and the signs of toxæmia are more pronounced. The development of a chronic stage is not uncommon, but on recovery some cases become fæcal carriers.

Bacillary dysentery was at one time widespread and extensive epidemics were common, but at the present time the largest epidemics are encountered in tropical and Eastern countries. There appears to be a close relationship between the presence of flies and the incidence of epidemics; cases are most numerous when flies are most abundant. Widespread outbreaks, mainly of the Flexner type, were reported in most theatres during the recent Great War.

Dysentery is endemic in this country; sporadic cases and small outbreaks occur frequently. Carriers are the usual source of infection and the main vehicle of infection is food, which becomes contaminated by handling. This type of infection is relatively frequent in asylums and has been termed "Asylum dysentery". It is, however, essentially the same as other endemic cases of the disease.

There is a definite geographical distribution of the various species of dysentery bacilli in Europe. Shiga strains are common in the south and east, while Flexner and Sonne strains are found in the north and west.

Diagnosis. The bacteriological diagnosis of dysentery is carried out by an examination of both the fæces and the blood.

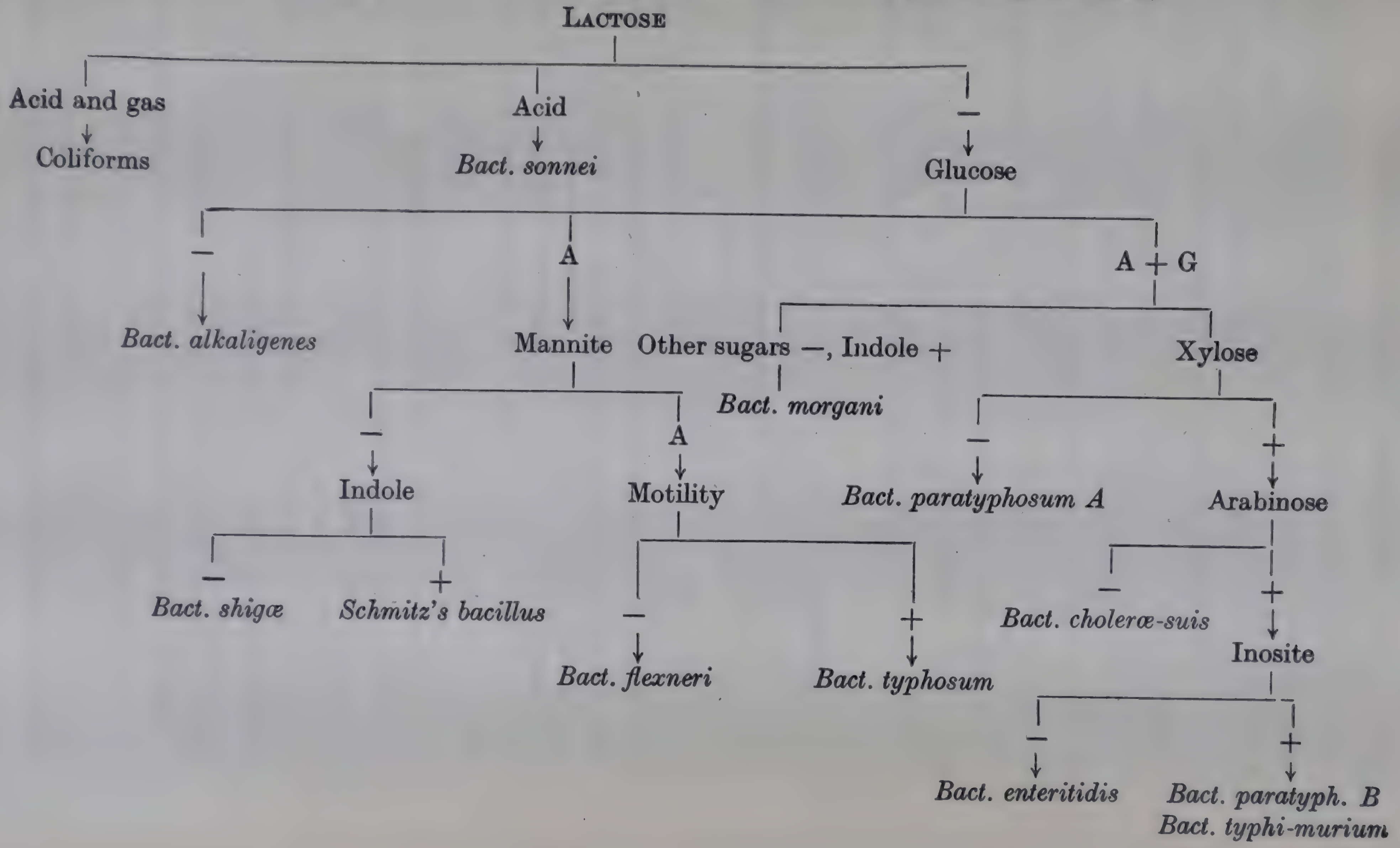
Cultural tests of fæces give the most valuable results, but useful information may be obtained by direct macroscopical and microscopical examinations. On macroscopical examination the fæces are usually fluid, containing much mucus and some blood. In bacillary dysentery during the first 24 hours much mucus with a few blood corpuscles is seen on microscopical examination. During the next 24 hours the red corpuscles are more numerous, and a large number of pus cells appear. The pus cells persist and increase in number during the next few days. This is in contrast with the amœbic form, in which the amœbæ may be seen but pus cells are frequently absent.

The cultural examination should be carried out as early as possible, as the chances of success decrease with the duration of the condition. The usual media, such as MacConkey and litmus lactose agar, only give satisfactory results during the early and acute stages of the disease. Two media, recently introduced, have a definite selective action on dysentery bacilli ; one contains sodium desoxycholate and citrates as the inhibitory substances and the other potassium tellurite, citrate and rosolic acid. Rectal swabs, with the desoxycholate-citrate medium, have proved valuable particularly in the search for carriers.

An examination of the blood for the presence of agglutinins may be made but the results are frequently equivocal. As previously stated, cultural tests of the blood are almost always negative. Agglutinins do not appear before the seventh day and therefore agglutination tests carried out in the early stages of the disease are of little value. The serum should be tested against various standard suspensions, *e.g.*, *Bact. shigæ*, various strains of *Bact. flexneri*, *Bact. sonnei* and Schmitz's bacillus. These organisms, being non-motile, contain only O or somatic agglutinogens. Agglutination consequently takes place slowly, and the tests should be left in a water-bath at 55° C. for at least 4 hours.

TABLE XIX

Abbreviated Biochemical Chart of the Bacterium Genus (Gram-negative Rods)


LACTOSE
Acid and gas
Coliforms
Acid
Bact. sonnei
−
Glucose
−
Bact. alkaligenes
A
Mannite
A + G
Other sugars −, Indole +
Bact. morgani
Xylose
−
Indole
−
Bact. shigæ
+
Schmitz's bacillus
A
Motility
−
Bact. flexneri
+
Bact. typhosum
−
Bact. paratyphosum A
+
Arabinose
−
Bact. choleræ-suis
+
Inosite
−
Bact. enteritidis
+
Bact. paratyph. B
Bact. typhi-murium

The interpretation of the results sometimes presents some difficulty, as normal serum may contain agglutinins for the dysentery bacilli. The titres in such cases are, however, low, and, in the case of *Bact. shigæ*, a reaction of 1/25 is suggestive and one of 1/40 is considered to indicate infection. With *Bact. flexneri* more difficulty is experienced, as a titre of 1/150 may be obtained with a normal serum. A titre of 1/150 or more is considered to suggest infection with *Bact. flexneri*. A titre of 1/100 with *Bact. sonnei* may also be given by the sera of normal individuals. In view of the difficulty in interpreting the results, agglutination tests are now seldom used as a routine procedure for the diagnosis of bacillary dysentery.

In Shiga infections agglutinins for *Bact. flexneri* may also be present in the serum; the converse, *i.e.*, the presence of agglutinins for *Bact. shigæ* in Flexner infections, does not, however, hold.

Prophylaxis. Measures adopted to prevent dysentery, as in the Salmonella group, are general and specific. In general prophylaxis attention is directed to sanitation, personal hygiene, destruction of flies and the control of carriers.

Specific measures involve the use of vaccines. In vaccines of *Bact. flexneri* all the various strains must be included, so that each antigenic component is adequately represented. The preparation is simple, cultures are killed by heat at 56°–60° C. for 1 hour and preserved by the addition of small amounts of phenol. A suitable dosage is 250, 500 and 500 millions at weekly intervals. They have not proved very satisfactory and are seldom used.

The preparation of Shiga vaccines is more difficult owing to the presence of the toxin produced by this organism. Treatment of cultures with formalin, in a similar manner to the preparation of toxoids, has proved useful. Cultures treated with 0·5–1 per cent. formalin for 15 days at 37° C. have a greatly reduced toxicity without any impairment of the immunogenic activity, and so large doses may be safely administered. Administration of large doses of the vaccine by the mouth has also been tried and satisfactory results have been claimed by some workers.

Therapy. The value of serum-therapy in dysentery has been the subject of much controversy; the results obtained in Flexner infections have been unconvincing, but the use of antitoxin in Shiga infections appears to be more satisfactory. This antitoxin is readily produced by the injection of horses with the specific toxin. The titration of this serum has presented some difficulty, and it is probable that some of the failures of serum-therapy might have been due to the use of a weak and unsatisfactory serum. The method of titration adopted by the International Standardization Commission consists in the intravenous injection of mice with dilutions of the serum plus a constant dose of a potent toxin. A comparison is made with mice injected with the similar doses of toxin and 1 unit of the standard serum.

The best results are obtained by early administration of the serum in relatively large doses by the intravenous route. An improvement in the clinical condition usually follows.

Chemotherapy. The sulphonamides have proved very successful in the treatment of bacillary dysentery ; the derivatives most widely used are sulphaguanidine and succinyl-sulphathiazole, both of which are not readily absorbed. Large doses are necessary, usually more than 50 grammes. The clinical response is good but, in the case of Sonne infections, there is not infrequently failure to clear the infection even after heavy dosage ; *i.e.*, the bacteriological response may be poor.

Summer Diarrhœa

A clinical condition closely related to dysentery is summer diarrhœa, which occurs in epidemic form frequently but not exclusively in children during the summer and autumn months. It was at one time a widespread and serious disease, but the incidence has decreased considerably in recent years as a result of improved hygienic conditions.

The disease was mainly encountered in the poorer districts, where overcrowding and unhygienic surroundings existed. Observations on the epidemiology of the condition in Manchester by Niven in the early years of the present century indicated that there was a close relationship between the prevalence of flies and the incidence of the disease. These observations have been confirmed by many workers.

In spite of the great incidence of summer diarrhœa, its ætiology remains unsettled. Different organisms have been incriminated in various countries. In France *Proteus vulgaris* was isolated from the stools of these cases, in America *Bact. flexneri* and in this country *Bact. morgani ; Bact. sonnei*, *Cl. welchii* and *Ps. pyocyanea* have also been isolated in other regions. Each of these organisms has been claimed as the ætiological agent of the disease. This suggests that several distinct organisms may be capable of producing the clinical condition known as summer diarrhœa. The seasonal incidence is due largely to the prevalence of flies and the greater possibility of bacterial multiplication in milk and various foods during the hot months. Summer diarrhœa is much less frequent in breast-fed than in artificially fed infants.

Prophylaxis. Improvement in general hygiene has reduced the incidence of the disease. Important factors in this respect have been the destruction of flies and the cleaning up of possible breeding places, such as manure-heaps and garbage.

CHAPTER XIX

THE FRIEDLANDER GROUP AND GENUS PROTEUS

THE organisms of the Friedlander group are Gram-negative, non-sporing rods, forming well-marked capsules, giving a mucoid growth on solid media and fermenting carbohydrates with the production of acid and gas. They are parasites frequently present in the upper respiratory tract of man.

The first member was isolated by V. Frisch in 1882 from a case of rhinoscleroma, and in the following year Friedlander obtained a similar organism from the lungs of fatal cases of pneumonia. This latter organism is usually known as Friedlander's bacillus or *B. pneumoniæ*, and is the most important member of the group. Other organisms possessing the characteristics of this group, *e.g.*, *B. ozœnæ*, isolated from cases of ozœna, have since been described, but they are relatively unimportant. The whole group is often discussed under the general term, *B. mucosus capsulatus*.

The taxonomic position of these organisms has not yet been determined. The usual practice is to regard them as closely related to, yet distinct from, the genus *Bacterium*, from which one of the main points of differentiation is in the formation of a well-marked capsule.

Morphology. The organisms are usually seen as short thick rods, $0{\cdot}6 \times 1\text{–}2\mu$, but they may exhibit pleomorphism and occur as clubs, filaments or long rods; they may also occur singly, in pairs or in short chains. On fresh isolation capsule formation can readily be demonstrated, and this tends to persist on artificial cultivation if satisfactory media are employed.

The organisms are non-motile and non-sporing. They stain readily with the ordinary dyes, but are decolorized in Gram's method and are Gram-negative.

Cultural Characteristics. Friedlander's bacillus grows readily on ordinary media. It is a facultative anaerobe and multiplies over a wide range of temperature; the optimum temperature is 37° C.

In *fluid media* moderate turbidity and a slimy sediment are formed.

On *solid media*, such as agar, a luxuriant, greyish, viscid growth is obtained. On blood-agar, hæmolysis is not produced but the plate is browned.

With the loss of the capsule, the type of growth alters and resembles more closely that formed by the genus *Bacterium*.

Resistance. Friedlander's bacillus is readily destroyed by bactericidal agents, being killed by heat at 55° C. in ½ hour. Stock-cultures can be conserved at room-temperature for several months.

Biochemical Activity. The saccharolytic activity is extremely variable. Acid and gas are formed from a number of sugars, usually glucose, mannite and maltose; lactose and saccharose give irregular results. Litmus-milk is usually acidified with, or without, clotting. Tentative attempts have been made to subdivide this group by the fermentation reactions.

With regard to proteolytic action, indole is not formed and gelatin is not liquefied. The growth in a gelatin stab culture presents a characteristic nail-like appearance; a rapid mucoid growth occurs at the surface, representing the head of the nail, with a line of growth down the stab.

Serology. Many attempts have been made to subdivide this group by serological tests. The most convincing of these is that introduced by Julianelle (1926), who obtained from capsulated organisms three sharply defined types and one heterogeneous sub-group; the rough forms were non-capsulated and had lost their type-specificity. The earlier work was doubtless handicapped by lack of knowledge of the antigenic structure and is consequently of uncertain value. Avery and Heidelberger isolated a polysaccharide, S.S.S., from the organisms and showed that two antigenic factors were present in the normal or smooth form—(1) a specific carbohydrate which, in certain types (B), was closely related to the S.S.S. obtained from type II pneumococci, and (2) a species-specific nucleoprotein.

Pathogenicity. These organisms are members of the normal flora of the upper respiratory tract, but they are frequently isolated from infections of this region. Friedlander's bacillus was first isolated from a case of pneumonia and was then considered to be the main ætiological agent of this disease. It was, however, later found that it was responsible for only a very small number of pneumonia cases, some 4–6 per cent. These cases are extremely severe and frequently fatal. Friedlander's bacillus has also been found in bronchitis, inflammation of the antrum and nasal sinuses, empyema, otitis media, meningitis, cystitis and peritonitis. It is also pathogenic to laboratory animals when injected intravenously, subcutaneously and intraperitoneally; injected subcutaneously, a local abscess is formed followed by septicæmia.

Prophylaxis and Therapy. Friedlander's bacillus is usually included in stock vaccines employed in the prophylaxis and therapy of infections of the upper respiratory tract.

Genus Proteus

The chief characteristics of this genus are: highly pleomorphic rods, Gram-negative and actively motile, forming a spreading growth on moist media; actively proteolytic; fermenting some carbohydrates with the production of acid and gas.

The members of this group were first described in detail by Hauser in 1885, three species being included in the classification—*P. vulgaris*, *P. mirabilis* and *P. zenkeri*. This sub-division has since been modified. Although motile and forming a spreading growth on solid media, *B. zenkeri* is Gram-positive and has little biochemical activity; it has therefore been separated from the genus proteus and placed in the separate genus, *Zopfius*. As members of the genus *Zopfius* are non-pathogenic, a further consideration of this group is unnecessary.

Morphology. The proteus organisms are very pleomorphic, short and long rods, filaments and curved forms are frequently present in the same field. They are actively motile and are Gram-negative.

Cultural Characteristics. Growth occurs readily on the ordinary media over a wide range of temperature. In *fluid media* a moderate turbidity with a slight deposit is usually formed in 24 hours. On *solid media*, such as agar, a phenomenon, termed "swarming", is frequently found; a thin growth spreads rapidly over the surface of the medium and is difficult to distinguish from the medium. A characteristic unpleasant smell is also produced. The property of swarming is due to the active motility of the organisms. On blood-agar the "β" type of hæmolysis occurs.

Resistance. These organisms behave as most non-sporing forms and are relatively susceptible to destructive agents. Stock cultures may be conserved at room-temperature for several months.

Biochemical Activity. The members of this group exhibit marked proteolytic activity. Gelatin is rapidly liquefied, urea is decomposed, but indole is not formed by all strains. Saccharolytic activity is also marked ; glucose, galactose and saccharose are usually fermented with the production of acid and gas ; lactose and mannite are never fermented, while the action on maltose is variable ; the fermentation of maltose and the formation of indole have been employed as a basis of sub-division of the group. Two sub-groups have been tentatively formed, one fermenting maltose and producing indole and the other being non-maltose fermenters and non-indole producers. Litmus-milk is reduced, and the casein may be digested with or without preliminary coagulation.

Serology. Attempts have been made to sub-group the proteus organisms by means of agglutination tests, but the results have not been very satisfactory. Being motile, both O and H antigens are present. This group, however, appears to contain a serologically heterogeneous collection of organisms. One strain, X 19, is employed in the Weil-Felix reaction, as it is agglutinated by the serum of typhus patients. The reason for this agglutination is unknown as the organism is not concerned with the ætiology of typhus fever. The reaction has, however, proved of great diagnostic value.

Pathogenicity. Members of the *Proteus* group are normally present in soil, putrefying material and sometimes in the fæces. They are pathogenic to man and may be responsible for cystitis and occur as secondary invaders in wounds, bronchiectasis and empyema. *Proteus* organisms are insensitive to penicillin and they may be responsible for persistence of infection or even secondary infection during penicillin therapy. They have also been incriminated by French workers as the ætiological agent of summer diarrhœa. The relationship of the species, *Proteus* X 19. to typhus fever is discussed later (p. 428).

In laboratory animals abscesses, followed sometimes by septicæmia, are produced on subcutaneous inoculation.

CHAPTER XX

PASTEURELLA : PLAGUE

THE characteristics of the genus *Pasteurella* are : Gram-negative ovoid rods, exhibiting bipolar staining, with slight biochemical activity and pathogenic to man and animals.

Members of this genus are responsible for a condition known as hæmorrhagic septicæmia in a large number of animals and birds. A specific name has been given to each of the various organisms according to the animal from which they were isolated : *Past. lepiseptica* from rabbits, *Past. oviseptica* from sheep, *Past. muriseptica* from mice, *Past. aviseptica* from fowls, etc. There is, however, little justification for such a system of classification and nomenclature. These organisms are closely related, and, as a precise method for their differentiation is not at present available, it would appear to be most convenient to consider them as members of one species, *Past. septica.*

One member of this genus, *Past. pestis* or the plague bacillus, is pathogenic in man. It was first isolated by both Kitasato and Yersin in 1894 from cases of plague. Pseudotuberculosis in guinea-pigs is caused by a pasteurella organism, which is usually termed *Past. pseudotuberculosis.* It differs from the other members of the group in that it is motile when grown at 22° C., but not at 37° C.

These various organisms have a number of similar characters, and, as *Past. pestis* is the member responsible for disease in man, it is the only one considered here in detail. It is important to note that organisms of the hæmorrhagic septicæmia group are common pathogens in most laboratory animals, producing a variety of lesions, and consequently they may be a source of irregularities in animal inoculation experiments.

Past. Pestis or the Plague Bacillus

Morphology. The plague bacillus is usually a small oval rod, $1{\cdot}5 \times 0{\cdot}6\mu$, with rounded ends and convex sides ; swollen involution forms are frequently found, particularly in old cultures and chronic lesions. Pleomorphism is particularly marked when growth is obtained on agar containing 3–4 per cent. NaCl, this constitutes an important diagnostic criterion. It is non-motile, having no flagella, and does not form spores. When freshly isolated from animal tissues a viscid capsule can be demonstrated.

Past. pestis stains readily with the ordinary aniline dyes and is Gram-negative. The stained forms show a definite bipolar reaction; this is not due to the presence of granules, but to the fact that the intervening protoplasm is usually less intensely stained than the polar areas. Methylene-blue is the most suitable stain for demonstrating this uneven staining.

Cultural Characteristics. Growth is obtained on the ordinary media, but it is slow and not very profuse; the optimum temperature is 30°–35° C., and the optimum reaction of the media is acid, pH 6·2–7·0; for primary isolation from the tissues enriched media, such as blood-agar or serum-agar, give the most satisfactory results. The plague bacillus is a facultative anaerobe, but growth under strictly anaerobic conditions is poor.

In *fluid media*, such as broth, after 24 hours' incubation there is a slight general turbidity with a definite floccular deposit, while later a surface pellicle may form. When sterile oil is floated on the surface of the medium, growth occurs downwards from the under surface of the oil in the form of stalactites.

On *solid media*, such as agar, small, transparent, colourless granular, viscid colonies are formed in 24 hours; later these become opaque, greyish, irregular, and tend to be differentiated into peripheral and central zones.

Resistance. The plague bacillus is readily killed by destructive agents; it is destroyed by heat at 55° C. in 5 minutes and by 0·5 per cent. phenol in 15 minutes. Stock cultures may be conserved at room-temperature or in the refrigerator for several weeks.

Biochemical Activity. The saccharolytic and proteolytic activities of the plague bacillus are not very marked. Acid, but not gas, is produced in glucose, maltose, rhamnose, glycerol and mannite after incubation for several days. As growth is poor in peptone water sugar solutions, Hiss's serum-water is a more suitable medium. Litmus-milk is not altered. Gelatin is not liquefied, and indole is not produced. Broth is rendered alkaline.

Serology. All strains of *Past. pestis* are serologically homogeneous and are agglutinated by the serum prepared against one strain. The serology of the group as a whole has not been completely investigated; cross agglutination is marked with

Past. pseudotuberculosis, but it is not so definite with the hæmorrhagic septicæmia group.

Pathogenicity. *Past. pestis* is primarily a pathogen in rodents, particularly wild rats, from which infection is conveyed to man through the agency of fleas, giving rise to a condition known as plague.

Experimental infection may readily be produced in a number of laboratory animals, *e.g.*, rats, mice, guinea-pigs, rabbits and other rodents. In all, after subcutaneous inoculation a necrotic local lesion and enlarged regional glands are first produced with subsequent septicæmia and death. The guinea-pig may also be infected by nasal instillation of the organisms or by rubbing them on the shaved skin of the abdomen.

Plague

Plague has been one of the most devastating diseases in the history of the human race. Reports of extensive and widespread epidemics have appeared in the literature of all ages. One celebrated epidemic, the "Black Death", swept this country and Europe in the fourteenth century and was responsible for the death of over 25 million people. At the present time the disease is rare in civilized communities, but large outbreaks are still reported in the East, particularly in India.

The isolation and incrimination of the plague bacillus were carried out independently by Kitasato and Yersin in 1894 during an investigation of an epidemic in Hong Kong. Subsequently several workers postulated that plague was essentially a disease of rats and that the spread to man occurred through the agency of fleas. This theory, however, was only conclusively proved by the work of the English Plague Commission at Bombay in the early part of the present century. Other rodents may also be responsible for outbreaks; epidemics in California have originated from the ground-squirrel, while in Tibet the marmot has been the primary source of infection in several outbreaks.

Epidemics have usually originated from four main endemic foci, two of which are located around the Himalaya mountains, one in Arabia and the other in Africa. In these regions plague is present in the rat population either in endemic form or as an epizootic. The rats involved are *Rattus rattus*, the small black house rat, and *R. norvegicus*, the large grey sewer rat. During an epizootic in these animals there is a plentiful supply of infected material, and it has been observed that human epidemics usually occur a short time after an epizootic in the rat.

The plague bacillus is conveyed from rat to rat and from rat to man almost exclusively by the rat fleas, particularly *Xenopsylla cheopis* and *Ceratophyllus fasciatus*. These fleas suck the blood of the rat and when the rat dies they search for another suitable host, rat if available, or, if not, man. Plague in its acute form is a septicæmic disease and the blood sucked up by the fleas consequently contains many bacilli, which multiply in the proventriculus and impede access of food to the stomach. The hungry flea settling on a fresh host sucks blood, but this further distends the œsophagus and regurgitation of the contaminated contents into the tissues of the host takes place. In other cases the plague bacilli may be implanted on the skin with the fæces or blood of the flea ; they usually gain entrance to the tissues as a result of subsequent scratching of the bite. Fleas once contaminated may remain so for some time, usually 7–15 days. Survival of the bacillus is favoured by a low temperature, about 50° F., and an almost saturated atmosphere.

In man two types of infection occur, (1) bubonic plague and (2) pneumonic plague. Of these the former is the more common condition. In bubonic plague, the bacilli are introduced by the flea into the tissues. After an incubation period of 5–7 days, the cardinal symptoms are painful enlargement of the regional glands, the so-called " buboes ", depression and prostration, with pyrexia. The local lesion may be extremely slight or negligible, but enlargement of the regional glands is invariably encountered. As people are usually bitten on the legs, the inguinal glands are frequently involved. In unfavourable cases septicæmia and death supervene ; this sequence of events is found in 60–90 per cent. of cases. Bubonic plague is only spread by the flea, direct infection does not occur. While the usual sequence is rat—flea—man, the cycle man—flea—man is also encountered.

Pneumonic plague does not depend on the presence of fleas for its transmission ; it spreads from man to man by droplet infection, and is usually found in the cold seasons. The primary focus in man appears to be those cases of bubonic plague in which pneumonic symptoms develop. The incubation period is short, 1–3 days, and the clinical signs are those of a fulminating bronchopneumonia. Septicæmia and death almost invariably result, the mortality rate being approximately 100 per cent.

Diagnosis. Bacteriological methods employed in the diagnosis of plague in man are the detection and isolation of the organisms from the various lesions, particularly the local vesicle, buboes, blood-stream and the sputum in the pneumonic form. Serological tests are sometimes used; agglutinins may be present in the serum to a titre of 1/10–1/100; a negative test is, however, valueless.

The diagnosis of plague in the rat is not infrequently a procedure of great importance. In this animal the disease may be either acute or chronic. In the acute form the usual lesions are enlarged lymph glands or buboes, mottling of the liver, pleural effusion, congested spleen and subcutaneous congestion and hæmorrhages. The plague bacilli are most readily isolated from the buboes, spleen and blood. In chronic cases, buboes and abscesses in the spleen and sometimes the liver are common. Except in the presence of epizootics the isolation of the organism is required in order to establish the diagnosis.

It is important that the presence of *Past. pseudotuberculosis* should be excluded, as this organism also gives rise to infection in rats. Important differential tests are the motility of this species at 22° C. and its lack of pathogenicity for white rats. Criteria useful in establishing the identity of *Past. pestis* are morphology, cultural characteristics, agglutination and pathogenicity for white rats.

Prophylaxis. Measures adopted in the prevention of plague are general and specific. The general measures employed in endemic foci include the suppression of rats, improvement in sanitary arrangements, removal of overcrowding and attention to personal hygiene, particularly to the avoidance of fleas. In the case of pneumonic plague, risk of infection must be avoided.

In countries where the disease is absent or extremely rare, the entrance of infected rats must be prevented and regular bacteriological examinations of the local rat population performed. This is one of the important duties of the Port Medical Officer. Quarantine regulations and the control of incoming ships are strictly enforced. Great precautions are taken with ships arriving from ports where plague is endemic; medical inspection on arrival is carried out, shields are placed on the hawsers and dead and trapped rats are submitted for a bacteriological examination. If a rat is reported infected, fumigation of the ship is at once carried out in order to kill the rats. It is largely as a result of the precautions taken by the port authorities that in this country plague is now a rarity.

Specific prophylaxis by means of vaccines has been extensively employed in Eastern countries. The usual method of preparation is to heat at 60° C. for 1 hour a 2–6 weeks' culture of *Past. pestis* grown at 25°–30° C. in digest broth, 0·5 per cent. phenol being added as a preservative. Unfortunately available statistics are incomplete, but the indications are that, by the use of vaccines, the incidence and mortality rate of the disease have both been reduced. Nevertheless, an entirely satisfactory vaccine has not yet been discovered. Schutze found that cultures incubated at 37° C. gave more protection to rats than those incubated at 26° C. At 37° C. a well-marked gelatinous envelope formed, whereas at 26° C. its development was poor. The presence of this envelope was considered to be important in the preparation of an efficient vaccine, as it contained an antigen quite distinct from that contained in the body of the organism. The envelope antigen was destroyed by heat at 100° C. The function of this envelope in the immunization of man has, however, not yet been determined.

Therapy. Specific therapy by means of sera prepared by various methods, *e.g.*, the injection of bacterial extracts and filtrates, has only been employed on a small scale, and it is impossible to arrive at any final conclusion of its value.

CHAPTER XXI

BRUCELLA: UNDULANT FEVER: TULARÆMIA

THE principal features of this genus are: Gram-negative minute rods with many coccoid forms, non-motile and non-sporing; aerobic or micro-aerophilic; carbohydrates not fermented; parasitic and pathogenic for man and animals.

The first member was isolated by Bruce in 1887 from cases of undulant fever at Malta, and was named *Micrococcus melitensis*. It was subsequently found to be a parasite and a frequent pathogen of goats. Bang in 1897, working in Denmark, obtained a minute organism from the uterine discharges of aborting cows; this he termed *Bacillus abortus*. A similar organism was later isolated from swine. These organisms were considered to be separate and distinct from *Micrococcus melitensis*, until Evans in 1918 demonstrated that a close morphological and cultural similarity existed between them. There was, however, a serological distinction; this has, however, recently been found to be less marked than the earlier work suggested. The indications from recent work are that the three organisms are essentially the same and have a common origin. As a result of adaptation to the different animal species—goat, cow and pig—the individual peculiarities have developed, giving rise to the caprine, bovine and porcine strains respectively.

The nomenclature and taxonomic position of this group have been rather confused and unsettled. Some workers have suggested the generic title "Alkaligenes," as the failure to ferment carbohydrates indicated a relationship with *Bact. alkaligenes*. This has, however, little to recommend it. *Bact. alkaligenes* has little in common with these organisms as it is a large organism with a low order of pathogenicity. Meyer and Shaw (1920) proposed for these organisms the generic name "Brucella" in the family "Bacteriaecæ" in honour of the discoverer of *M. melitensis*. This name is now generally accepted. Meyer and Shaw included two members, *Br. melitensis* and *Br. abortus* (bovine and porcine).

Other species, that do not conform in all details with the generic description, have been included in this group by many workers, viz., *Br. bronchiseptica* and *Br. tularensis*. This arrangement is only tentative and may require modification in the future. *Br. bronchiseptica* was isolated independently by Ferry and McGowan in 1911 from cases of canine distemper. It was then considered to be the primary agent of this disease, but it is now accepted as a secondary invader. It differs from the generic description in that it is motile.

Br. tularensis was obtained by McCoy and Chapin in 1912 from a septicæmic disease in the ground-squirrels of California. Its cultural, morphological and serological reactions bear a close relationship to the Brucella group. Some workers, however, consider that, in view of the nature of the disease, it should be included in the Pasteurella genus.

Morphology. Members of the *Brucella* group are minute rods, 0·6–1·5 × 0·6μ, appearing as cocco-bacilli and without any characteristic arrangement. *Br. melitensis* is frequently more coccal than *Br. abortus*. With the exception of *Br. bronchiseptica*, they are non-motile. All are non-sporing and do not form demonstrable capsules.

They stain readily with the ordinary stains, are Gram-negative and frequently show bipolar staining.

Cultural Characteristics. Growth occurs readily on the ordinary media, but it develops slowly and is never profuse. The optimum temperature is 37° C. and the optimum reaction *p*H 7·2–7·4.

In all cases growth in the absence of oxygen is either absent or scanty. *Br. abortus* differs from the other members in that, on first isolation, the presence of 5–10 per cent. CO_2 in the atmosphere is usually essential and is always favourable for growth. After several sub-cultures on artificial media growth occurs readily under the usual atmospheric conditions. In *fluid media*, such as broth, after 24 hours' incubation at 37° C., a slight turbidity forms ; on further incubation a moderate turbidity and light deposit are found.

On *solid media* such as agar, small, greyish, semi-opaque colonies are formed in 24–48 hours at 37° C. On potato a yellowish-brown pigment is produced.

Resistance. The *Brucella* organisms are readily destroyed by most bactericidal agents ; they are killed by heat at 60° C. in 10–15 minutes. There is, however, some variation in the behaviour of the different species to the bacteriostatic action of certain dyes. The effect is tested by adding the dyes to liver-infusion agar to give a final dilution of 1/25–50,000 of thionin and basic fuchsin and 1/100,000 of methyl violet.

The bovine strain of *Br. abortus* is inhibited by the thionin, but not by the fuchsin or methyl violet. The porçine strain of *Br. abortus* is inhibited by both basic fuchsin and methyl violet, but not by the thionin. *Br. melitensis* is not inhibited by any (Table XX.).

Biochemical Activity. Members of this group exhibit little biochemical activity. Carbohydrates are not fermented, indole is not produced and gelatin is not liquefied. H_2S is formed by *Br. abortus*, but not by *Br. melitensis*.

Serology. Serological methods have not proved satisfactory as a means of differentiating members of this genus. Discordant results have been obtained by many workers. Recent work has, however, found an explanation for the irregularities. When freshly isolated the organisms are in the smooth form and are agglutinated only by sera prepared against smooth strains. After artificial cultivation for a variable period, a few days to several years, they become rough and are not agglutinated by the smooth antisera. Strains in the transition stage from smooth→ rough may exhibit agglutination with both smooth and rough antisera. Failure to differentiate smooth from rough strains had been largely responsible for the confusion of the earlier workers.

Smooth strains were found to be not thermo-agglutinable, and the difference in the behaviour of *Br. melitensis* and the bovine and porcine strains of *Br. abortus* to be of a quantitative and not qualitative nature. The same two main antigen components, styled " A " and " M," are possessed by these organisms, one being major to the other in their respective cases. By quantitative absorption methods it is possible to obtain a serum which is practically mono-specific.

Rough strains are thermo-agglutinable, as tested by heating a saline suspension at 90° C. for 1 hour, and are closely related to each other. They correspond to the " para-abortus " and " para-melitensis " strains, which had been described by some bacteriologists.

Pathogenicity. These organisms produce a spontaneous disease in goats, cattle and pigs, from which infection is conveyed to man. The infectivity of *melitensis* strains for man is high. On the other hand, it would appear that *abortus* strains have only a low degree of infectivity. Frank cases of *abortus* infection are relatively rare considering the possibilities of infection, but there is no doubt that sub-clinical and extremely mild attacks are very common, as judged by the presence of agglutinins in the serum of healthy individuals, particularly in individuals coming in frequent contact with cattle.

Experimentally an infection sometimes resulting in death can be produced in the guinea-pig ; porcine strains of *Br. abortus* are the most and bovine strains the least virulent for these animals. In the monkey it appears that melitensis and porcine abortus strains are definitely more infective than bovine abortus strains.

In man melitensis and porcine strains are more virulent than bovine strains ; *Br. melitensis* is responsible for the condition known as Malta, Mediterranean or undulant fever ; the porcine and bovine strains of *Br. abortus* give rise to the so-called "abortus fever". The mode of infection and the disease process of these conditions appear to be essentially the same, and they are both considered as undulant fever.

Undulant Fever

Undulant fever was originally found mainly in Malta and the countries bordering the Mediterranean Sea, and it was consequently known as Mediterranean or Malta fever. Its prevalence in Malta led to the formation in 1904 of a special commission to investigate it. As a result the *rôles* played by the goat as the primary focus of infection and by goat's milk as the vehicle were determined. The subsequent restriction and control of the consumption of goat's milk enormously reduced the incidence of the condition.

It was not until the relationship of the *M. melitensis* of Bruce and the *B. abortus* of Bang was established by Evans in 1923 that animals other than the goat, particularly the cow, were considered to be responsible for the disease. These observations were soon confirmed and recent investigations have shown that the disease is widespread ; cases have been reported in many countries. It has probably existed in this country as an unrecognized disease for some time ; *Br. abortus* is the common ætiological agent and the usual vehicle of infection is cow's milk. Infection may also follow the handling of infected material, such as milk and meat. The handling of infected meat is the usual mode of infection with porcine strains of *Br. abortus*.

The incubation period is uncertain ; most observers give from 8–14 days as the time of onset after the ingestion of the organisms. The symptomatology of the disease is extremely variable; headache, lassitude, general malaise, bone pains, profuse sweating and pyrexia of the intermittent type are usually present. The chief points of these infections are the long and indefinite duration, the irregular course and the low mortality rate. The course is characterized by a series of febrile attacks, each lasting about a week, and the average duration of the disease is approximately 90 days; in some cases the fever has lasted as long as 300 days.

Infection with *Br. abortus* usually resembles the milder types of *melitensis* infections, but the clinical picture is equally variable, and fatal cases have been reported. It is interesting to note that abortion has never been reported in the case of female patients. The clinical picture may resemble that seen in enteric fever, malaria, tuberculosis, Hodgkin's disease and malignant endocarditis.

Diagnosis. The bacteriological diagnosis of undulant fever involves (1) the isolation of the organism, and (2) the detection of agglutinins in the serum.

(1) **Isolation.** The organism may be isolated from the bloodstream during a febrile attack either by primary cultivation in broth or by intraperitoneal injection of the guinea-pig. Both methods may be employed, but as direct cultures in the early stages are positive in approximately 80 per cent. of cases, animal inoculation tests are not usually required. The blood should be collected at the height of the fever and 5 c.c. seeded into two flasks containing about 100 c.c. of liver infusion broth; both are incubated for

TABLE XX

Differentiation of the Species of the Brucella *Genus*

Strain	CO_2 requirement	H_2S production	Growth in presence of		
			Thionin	Basic fuchsin	Methyl violet
Br. melitensis . .	—	—	+	+	+
Br. abortus (bovine) .	+	+	—	+	+
Br. abortus (porcine) .	—	+	+	—	—

several weeks at 37° C., one aerobically and the other in an atmosphere containing 10 per cent. CO_2.

Cultures may also be made of urine and material obtained by splenic puncture; urine culture is, however, generally of little value. In identifying the organism the various differential tests should be employed; these are given briefly in Table XX.

(2) **Agglutination.** Agglutinins to a high titre (up to 1/3,000) can frequently be demonstrated in the serum of patients; they usually appear about the fifth to eighth day. In certain cases the appearance of agglutinins may be delayed for 2 or 3 weeks. In carrying out the tests the bacterial suspensions must be prepared when the organisms are in the smooth state, otherwise the results are unreliable. The "*prozone*" phenomenon may be observed, *i.e.*, serum dilutions 1/20 and 1/40 may not exhibit agglutination whereas the higher dilutions show marked clumping.

The interpretation of the reaction may present some difficulty when a comparatively low titre is obtained. It has been found that the serum of individuals dealing with infected animals, such as veterinary surgeons, slaughterers, butchers, etc., may contain agglutinins to *Br. abortus* sometimes to a titre of 1/100. A titre of less than 1/100, particularly in individuals coming in contact with cattle, is therefore of little value in determining the presence of infection. The presence of a rising titre on repeated examination, however, suggests infection.

(3) **An Intradermal Test** with *abortin*, which is a filtrate of a 4–6 weeks' culture heated at 99° C. for 2 hours, has in some instances proved useful. In a positive test, which is an allergic phenomenon, a local reaction is seen within 24 hours.

Prophylaxis. The most valuable measure in the prevention of undulant fever is the control of the milk supply. In the case of melitensis infections the recognition of the *rôle* played by goat's milk and the subsequent banning of its consumption reduced the incidence of the disease in a striking manner. Figures collected at Malta among the Army and Navy population illustrate the considerable reduction in the incidence of the disease after the introduction of preventive measures (Table XXI.).

Measures were also introduced to suppress the disease in goats; in certain districts the percentage of infected animals was found to be very high and the eradication of the disease was consequently impossible.

The elimination of *abortus* infections presents a more difficult task. It is very prevalent in the cattle of this country; workers

TABLE XXI

Effect of Milk Control on the Incidence of Undulant Fever at Malta

Year	Cases	Deaths
1905 (no control)	913	23
1907 (control)	21	1

have found that in not less than 20–50 per cent. of all cows examined, agglutinins to a titre of 1/40 were present in the serum and, of these, some 10–30 per cent. were excreting *Br. abortus* in the milk. Prevention is thus a formidable problem, as banning the consumption of milk is not practicable. It is therefore necessary either to treat the milk or to eliminate infected animals.

In the treatment of milk pasteurization is the most useful procedure, as the organisms are readily destroyed at the temperature involved in the process. In London where this practice is widely adopted cases of the disease are practically unknown.

The detection of infected animals is assisted by various bacteriological tests, such as the examination of milk and serum for agglutinins and of the milk also for the presence of the responsible organisms. In the latter test selective media, such as liver-infusion agar containing 1 in 100,000 gentian-violet and with a *p*H of 6·6, and incubation in an atmosphere of 10 per cent. CO_2 are necessary; under these conditions pure cultures can be isolated without difficulty. Another and perhaps more certain method is the intraperitoneal or subcutaneous inoculation of a small amount of the milk into a guinea-pig, which is killed in 3–4 weeks, when cultures are prepared from various organs, such as the spleen, lymph glands and blood.

Vaccines have proved of doubtful value both in prophylaxis and therapy. Success has been claimed with the therapeutic application of abortin given intramuscularly, but it has not yet been tried on a large scale. Living organisms have been used for the immunization of cattle, but this is far from satisfactory, as the organisms tend to be excreted in the milk, thereby increasing the risk of infection in man.

Tularæmia

Tularæmia is primarily a disease of rodents and is encountered mainly in the ground-squirrels of California; it occurs as a fatal septicæmia and resembles plague in many respects. Man is very susceptible; infections have been reported in America following the handling of infected materials, such as ground-squirrels or wild rabbits, and laboratory infections have not been uncommon. Infection may also take place by inhalation or through the agency of blood-sucking insects. *Br. tularensis*, the ætiological agent, can either pass through the unbroken skin or gain entrance by way of minute abrasions.

The clinical picture of the disease in man is variable, and several clinical types have been described. In some cases a local skin lesion is present together with painful enlargement of the regional lymph glands; pyrexia and secondary constitutional disturbances are also present. In other instances, usually found in laboratory infections, the local lesion is not present. Signs of a vague generalized infection with intermittent pyrexia are found. This is known as the "typhoid" type.

The main diagnostic test is the examination of the serum for the presence of agglutinins. Cultures of the blood are almost invariably negative. The organism may sometimes be isolated by injecting guinea-pigs in series with infected material, such as blood or enlarged lymph glands.

CHAPTER XXII

HÆMOPHILUS AND ASSOCIATED ORGANISMS: WHOOPING-COUGH

Hæmophilus is strictly the generic name of certain Gram-negative rod-shaped organisms, essentially parasitic, that require for growth certain accessory factors which are present in blood and some plant tissues.

A typical member of this genus is the influenza bacillus or *H. influenzæ*, growth of which is only obtained when the accessory factors are present in the medium. This organism was first described by Pfeiffer, who isolated it in 1892 from the sputum of influenza cases and considered that it was the ætiological agent of the disease. Several organisms, however, not fulfilling all the generic growth requirements have since been included in this group by different workers. These possess many of the other generic criteria; they are Gram-negative, small, non-motile rods with limited biochemical activity, but, while growth is not easy to obtain, the presence of the accessory factors is not obligatory. The following species, pathogenic for man, are usually included in this group: Koch-Weeks bacillus, *H. pertussis*, Ducrey's bacillus of soft sore and the Morax-Axenfeld bacillus of angular conjunctivitis in man. In the last three cases there appears to be very little justification for this inclusion. Recent work indicates that there is a close relationship between *H. pertussis* and *Br. bronchiseptica*.

A number of hæmophilic organisms have recently been isolated from the naso-pharynx of normal individuals; these resemble in many respects *H. influenzæ* and have been loosely designated atypical or para-influenza bacilli. These strains are usually non-pathogenic in laboratory animals.

H. INFLUENZÆ (INFLUENZA OR PFEIFFER'S BACILLUS)

The influenza bacillus is a normal member of the upper respiratory flora, and it is not found in nature outside the body-tissues. It is an obligate parasite.

Morphology and Staining Reactions. The morphological appearance of the influenza bacillus not infrequently exhibits marked variation. The usual form is a minute rod, 1–1·5 × 0·25μ, which may tend to resemble cocci; in fact, they are often termed cocco-bacilli. In some cases, although the cocco-bacillary forms predominate, long bacilli may be found; in other instances long curved threads may be the main or only form. In view of this diversity of appearance, it has been suggested that the influenza bacillus might be sub-grouped on morphological grounds. This method of classification has not been generally accepted. One of the chief objections is that the morphology of a given strain is by no means constant. In one culture various forms, such as cocco-bacilli, large bacilli and filaments may be observed.

The influenza bacillus is non-motile and does not form spores or a readily demonstrable capsule.

It does not stain readily with the simple stains, of which dilute carbol-fuchsin gives the best results. It is Gram-negative.

Cultural Characteristics. As the generic requirements indicate, the influenza bacillus does not grow on the simple media. Growth occurs on blood-agar, but not on agar enriched with serum or ascitic fluid. The special requirements of this organism have been closely examined, and it has been established that in order to obtain growth the presence of two substances, which have been termed " accessory factors ", is essential. One of these has been designated the V factor and appears to be a coenzyme; it is present in blood and various vegetable tissues, especially yeast and potato, and is destroyed by autoclaving at 120° C. for 30 minutes. It is also produced by many organisms.

The other or X factor is present in blood and is the iron-containing hæmoglobin derivative, hæmatin. It is active in high dilutions, is not inactivated by heat at 120° C. in 30 minutes and is required for the synthesis of peroxidase and catalase.

Although both V and X factors are present, blood-agar in an unmodified form is not a satisfactory medium; its value may be greatly enhanced by various processes, which convert hæmoglobin into hæmatin and break up the corpuscles and diffuse their contents. Modified media, such as chocolate-agar and Fildes' medium, are consequently in frequent use. Chocolate-agar is prepared by heating the ordinary blood-agar medium at 99°–100° C. for 1–3 minutes; by such treatment the hæmoglobin and some of the corpuscles are disintegrated. Fildes produced similar effects by digesting the blood with pepsin before its addition to either agar or broth.

Growth is never very profuse, even on the most suitable media. The optimum temperature is 37° C. and aerobic conditions are required; the influenza bacillus is considered to be a strict aerobe.

In *fluid media*, a slight general turbidity is usually formed. The thread-like forms tend to give a flocculent deposit with little turbidity.

On *solid media*, minute, smooth, transparent, circular colonies are formed in 24 hours. Atypical strains, such as those showing threads, give colonies that are more opaque and have a bluish iridescence. Growth becomes more marked on further incubation and the colonial appearance tends to become irregular. When grown on a blood-plate with a *Staphylococcus* the colonies of *H. influenzæ*, growing around a staphylococcal colony, are usually larger than the discrete influenzal colonies. This phenomenon is termed "*satellitism*" and is due to the synthesis by the *Staphylococcus* of the V factor, which diffuses into the surrounding medium.

Resistance. The influenza bacillus is readily destroyed by bactericidal agents. It is killed by heat at 60° C. in 5 minutes. It is difficult to conserve; cultures should be kept at 37° C. and subcultures must be made every 5–7 days.

Biochemical Activity. The biochemical properties of the influenza bacillus have been inadequately studied mainly on account of its growth requirements. The difficulty in obtaining growth is also doubtless responsible for the discordant results obtained by various workers.

In the case of the fermentation reactions discrepancies have arisen, but many workers agree that acid, but not gas, is produced from glucose, maltose and lævulose by most strains. The proteolytic activity is very slight, but many strains are able to produce indole. Indole-formation appears to be a stable character, and this property has been used as the criterion for the subdivision of the influenza bacilli into two groups—the indole producers and the non-indole producers. The non-indole producing strains are often atypical, hæmolytic and avirulent to mice.

Hæmolysis is shown by some strains when grown on blood-agar plates. These hæmolytic strains usually do not require the X factor, they fail to form indole, and, on morphological examination, occur often as threads or long rods.

Serology. Serological studies of the influenza bacillus have been very unsatisfactory. Both agglutination and complement-fixation tests indicate that a marked heterogeneity exists among the various strains examined. Attempts at sub-division by such tests have consequently not proved fruitful. Some of the complications have doubtless been due to the failure of workers to differentiate between the smooth and rough strains. Smooth strains have recently been divided into two main groups but this work is still in the preliminary stages.

Toxin Production. It has been found that the intravenous injection of relatively large doses of filtrates of young cultures into laboratory animals results in marked prostration with death in 24–48 hours. The responsible substance, however, is not considered to be a toxin, as it is non-specific and non-antigenic; its exact nature is uncertain.

Pathogenicity. The influenza bacillus produces spontaneous disease only in man. It is, however, pathogenic to a number of laboratory animals. Nasal instillation under ether anæsthesia of virulent strains into mice and rabbits gives rise to a fatal broncho-pneumonia. Blake and Cecil (1920) instilled a culture of a virulent strain into the nose and mouth of 12 monkeys; all exhibited signs of infection and, in two, pneumonia developed.

The *rôle* played by the influenza bacillus in causing disease in man has been the subject of much controversy. When it was first isolated by Pfeiffer in 1892 it was accepted as the ætiological agent of influenza. Many later workers failed to confirm this view, and although an enormous amount of work has been done, particularly during the 1918–19 pandemic, the exact relationship of the organism to influenza remained unsettled until 1933. Laidlaw, Andrewes and Smith, employing ferrets as experimental animals, then found that, in epidemic influenza, a filterable virus is the primary agent and that the influenza bacillus is a secondary invader (see p. 409).

H. influenzœ may, however, be a definite pathogen for man. It is frequently a member of the normal flora of the upper respiratory tract, but when the local resistance is reduced it may become actively pathogenic. Many cases of broncho-pneumonia following influenza, measles, whooping-cough, etc., are caused by this organism, and in such cases the mortality rate is usually high. It may also be responsible for a number of other conditions, such as antrum infections, otitis media, empyema, endocarditis, arthritis, conjunctivitis, and meningitis.

Isolation. The isolation of Pfeiffer's bacillus from uncontaminated material, such as cerebrospinal fluid, is a relatively simple matter. Direct culture on to chocolate-agar, blood-agar and ordinary or serum-agar is usually sufficient. Growth is most marked on the chocolate-agar and absent on the ordinary or serum-agar. When the material is grossly contaminated, as in swabs or washings of the naso-pharynx, the isolation of the organism by this method becomes a much more difficult problem. Fleming and MacLean simplified this examination when they found that the addition of the filtrate of a broth culture of a mould (*Penicillium notatum*) to chocolate- or blood-agar inhibited the growth of many organisms. There was no effect on the influenza bacillus. Using this technique hæmophilic organisms have been readily isolated from the human naso-pharynx, but in many instances these organisms have probably been the avirulent, atypical influenza bacilli and not the true virulent species.

KOCH-WEEKS BACILLUS

The Koch-Weeks bacillus is so named because it was isolated from cases of acute catarrhal conjunctivitis first by Koch in Egypt and by Weeks in New York. This type of infection is common and widespread attacks may be encountered. The bacillus has been investigated by different workers, and the results indicate that it is identical with the influenza bacillus.

H. PERTUSSIS OR THE BORDET-GENGOU BACILLUS

H. pertussis was first isolated by Bordet and Gengou in 1906 from the sputum of whooping-cough patients. It was agglutinated by the patient's serum, with which it also gave positive complement-fixation tests, and was consequently considered to be the ætiological agent of whooping-cough.

Morphology. The morphological appearance of *H. pertussis* resembles that of *H. influenzæ.* Pleomorphism is less marked, the usual form is a short, thick, oval rod ; threads may appear in old cultures. It does not stain readily with the ordinary dyes, of which toluidin-blue gives the best results, and is Gram-negative. It is non-motile and non-sporing. Capsules have been observed, but their presence is not easily detected.

Cultural Characteristics. Growth is not readily obtained on primary isolation from the human tissues. Enriched media, such as blood-agar, are required ; the potato-extract glycerine blood-agar medium, which was introduced by Bordet and Gengou, is particularly satisfactory. After cultivation through several generations under artificial conditions, the Bordet-Gengou bacillus is able to grow quite easily on relatively simple media, such as serum-agar and even ordinary agar. Neither the X nor V factor is essential for growth, and the organism is consequently not strictly a member of the *Hæmophilus* genus.

H. pertussis is a strict aerobe and its optimum temperature for growth is 37° C.

On *solid media*, such as blood-agar, when first isolated, growth is slow and delicate ; the colonies are small and transparent after 24 hours, but on further incubation they become larger, greyish and opaque. After sub-culturing through several generations growth is more rapid and profuse, the colonies being greyish and opaque.

In *fluid media*, such as serum-broth, a uniform turbidity is produced, sometimes with a slight deposit.

Resistance. *H. pertussis* is readily destroyed by bactericidal agents ; it is killed by heat at 56° C. in 15–30 minutes. Cultures on potato blood-agar may remain viable for 3 weeks.

Biochemical Activity. Little biochemical activity is produced by the Bordet-Gengou bacillus. Indole is not formed, gelatin is not liquefied and carbohydrates are not fermented. Some strains produce a hæmolysin.

Serology. *H. pertussis*, when freshly isolated, has a homogeneous antigenic structure, and during an epidemic only one serological type is generally found. This is readily differentiated from the *H. influenzæ* group and is agglutinated by the serum of convalescents. There is, however, an important alteration in the antigenic structure during growth under artificial conditions. Four phases (I, II, III and IV) have been described ; phases I and II are smooth and toxic, while phases III and IV are rough and non-toxic. In the rough phases the organisms are not agglutinated by convalescent serum. Strains kept on media containing 25 per cent. blood remain in phase I for a long time. Reversion from the rough phases III and IV to the smooth phases may be brought about by cultivation on media containing a high percentage of blood.

Pathogenicity. *H. pertussis* was first isolated from cases of whooping-cough by Bordet and Gengou and was considered by them to be the ætiological agent of the disease. Although this view has been subjected to much criticism, it is accepted by the majority of workers at the present time. The condition cannot be reproduced in laboratory animals ; attempts to produce the disease in apes have been unsatisfactory. The intraperitoneal injection of large quantities of the organisms into guinea-pigs causes death in 24–48 hours. As a similar result is obtained with killed cultures, the lethal action is attributed to the activity of an " endotoxin."

Whooping-cough

Whooping-cough is a common and serious disease of childhood, cases being most numerous in infants under 5 years of age. It is transmitted either by direct contact or by droplet infection, and epidemics in schools or similar institutions are frequently encountered. The incubation period is very variable, from a few days to a fortnight, and the onset is characterized by cough, anorexia and general malaise. The cough is at first dry and short, but later becomes paroxysmal and may be followed by vomiting. During a paroxysm there is usually a series of rapid, dry coughs, followed by a violent inspiratory effort which produces the typical whoop. This stage may last for several weeks, and is followed by recovery or the onset of complications, the most important of which are broncho-pneumonia, bronchitis and convulsions.

The lesions are found in the mucosa of the upper respiratory tract, which is the seat of hyperæmia and catarrhal inflammation. It is considered that the cilia of the trachea become clogged and destroyed by mucus and the organisms, and these collect instead of passing upwards and are only expelled by the paroxysm of coughing. There may be absorption into the blood-stream of the endotoxins which may affect the central nervous system.

Diagnosis. The isolation of *H. pertussis* from cases of whooping-cough is not a simple matter. It is almost invariably present in the sputum during the first week of the illness, but then becomes less frequent and is rarely found after the third week. The isolation of the organism has been simplified by the use of potato-extract glycerine blood-agar and the adoption of the " cough-plate " technique. The plates are held close to the mouth during a paroxysm of coughing and are then incubated at 37° C. ; suspicious colonies are picked off after 24–48 hours.

Serological tests, *e.g.*, agglutination and complement-fixation, with the patient's serum may be employed after the third or fourth week ; in carrying out these tests the bacterial suspension must contain the organism in phase I or II.

Prophylaxis. Specific immunization by means of vaccines has been carried out in different countries. While the results are conflicting, several workers have claimed that vaccines not only protected against an attack, but that, if the disease developed, its course was modified.

The early results are of little value as the possibility of variation in the antigenic structure of the organism was not appreciated. It is now recognized that, in the preparation of vaccines, only organisms in phase I should be employed. A uniform dosage has not been used, it has varied from three injections of 250 to 5,000 million organisms on alternate days ; it appears, however, that repeated large doses are necessary to produce a satisfactory degree of immunity.

Therapy. Vaccines have also been employed in the treatment of the disease by various workers. It is claimed that, given in the early stages, vaccine therapy cuts short the attack. The results, however, are far from conclusive and many unsuccessful attempts have been reported.

THE MORAX-AXENFELD BACILLUS

This organism was described independently by Morax (1896) and Axenfeld (1897) and is responsible for a type of subacute conjunctivitis in man. It usually occurs as a short Gram-negative rod arranged in pairs or short chains, but in old cultures pleomorphism is frequently seen. It is non-motile, non-sporing and non-capsulated.

Growth occurs under aerobic conditions on media enriched with serum, ascitic fluid or blood ; the Morax-Axenfeld bacillus is consequently not a true member of the *Hæmophilus* genus. Its correct generic position has not, however, been determined.

On solid media, such as serum-agar, small, greyish, translucent colonies appear in 24 hours ; serum is liquefied. In fluid media a general turbidity is at first formed, but later a greyish deposit appears. It is non-pathogenic in animals, but on instillation into the healthy human conjunctiva a definite inflammatory reaction develops in 4–5 days.

THE BACILLUS OF SOFT SORE OR DUCREY'S BACILLUS

This organism was first isolated by Ducrey in 1889 by injecting material from a case of soft sore into the arm. After several arm-to-arm passages the pus yielded a pure culture of a Gram-negative bacillus, which is usually termed " Ducrey's bacillus ".

It is a short Gram-negative bacillus, and in pus tends to be arranged in pairs, side by side, and may be intracellular. It is non-motile, non-sporing and non-capsulated.

It does not grow readily in culture, and enriched media, such as blood-agar, are required. It is not, however, a true hæmophilic organism, as the X and V factors are not essential for growth. Its taxonomic position is as yet undefined.

It is as a rule non-pathogenic in laboratory animals, but soft sores have been produced by the inoculation of pure cultures into man and certain monkeys.

CHAPTER XXIII

CORYNEBACTERIUM: DIPHTHERIA

THE following definition has been given to the genus *Corynebacterium* by the Society of American Bacteriologists: slender, often slightly curved, rods with tendency to club formation, branching cells occasionally seen in old cultures; barred irregular staining; not acid-fast; Gram-positive; non-motile; aerobic; no endospores; some pathogenic species produce a powerful exotoxin.

Although Reymond isolated an organism, which appears to have been *C. xerosis*, from the conjunctival sac in 1881, the discovery of the diphtheria bacillus in the diphtheritic false membrane by Klebs in 1883 was of much greater importance. In 1884 Loeffler published his classical paper, in which he detailed the isolation of the diphtheria bacillus from the throats of diphtheria patients and the subsequent production of lesions in numerous experimental animals. These observations were repeatedly confirmed by various workers. Organisms resembling the diphtheria bacillus were also isolated from different situations and claims of their separate identity were made. In many instances there appears to have been little justification for these claims; this applies particularly to the various organisms isolated from the human naso-pharynx and styled loosely as *diphtheroids*. In some instances, however, there is no doubt that distinct species were isolated from man and animals. In 1888 Hofmann obtained an organism from the throats of normal individuals which was similar in many respects to the diphtheria bacillus. This is known as *C. hofmanni* or Hofmann's bacillus, and is the most important of the so-called diphtheroids occurring in man.

Sometime later, in 1896, Unna isolated *C. acnes* from the lesions of acne; its relationship to this condition is, however, uncertain. Members of this group are also important pathogens in animals: *C. pseudotuberculosis ovis* or the Preisz-Nocard bacillus was isolated in 1889 by Nocard and later by Preisz from the lesions of pseudotuberculosis in sheep; *C. pyogenes* was obtained in 1890 by Enderlen from a case of purulent pyelitis in a cow; *C. pseudo-*

tuberculosis murium was isolated from pseudotuberculosis in the mouse by Kutscher in 1894.

C. DIPHTHERIÆ OR THE DIPHTHERIA BACILLUS

As the diphtheria bacillus was first described by Klebs and isolated by Loeffler, it is sometimes referred to as the Klebs-Loeffler bacillus (K.L.B.).

Morphology and Staining Reactions. The morphological appearance of the diphtheria bacillus presents several characteristic features. It is consequently one of the few organisms where identification may be possible by microscopical examination alone. As a rule it is a straight or slightly curved rod, 3–5 × 0·5μ, but pleomorphism is frequently encountered. Short forms and cells swollen at one end, to represent clubs, in the centre or at both ends are frequently found.

The arrangement is also distinctive. Single forms and groups occur; in the groups the organisms tend to be arranged at angles to each other resembling the letters L or V; combinations of these are likened to Chinese or cuneiform writing. This peculiar arrangement is probably due to the method of division and the incomplete separation at the moment of division.

The staining reaction is uneven; in the same organism deep and light staining areas are seen. Also granules, first described by Babes (1886) and Ernst (1888), are frequently present, particularly in *mitis* strains; these are usually termed *metachromatic* or *Babes-Ernst* granules. They may be situated at the poles of the cell, when they are called "bipolar," or they may be scattered irregularly throughout the protoplasm. A single cell may contain anything up to half a dozen, but the usual number is two or three. In order to accentuate the granules and simplify identification, special staining methods are employed; the most useful of these are Loeffler's methylene-blue and Neisser's differential stain (see Plate II.).

The diphtheria bacillus is Gram-positive, but it is decolorized more readily than most Gram-positive organisms; the granules, however, retain the stain more tenaciously than the remaining protoplasm.

C. diphtheriæ is non-motile, having no flagella, and does not form spores or a demonstrable capsule. Branching has been reported; this is not seen in young cultures, and is considered by many observers to occur only in degenerate and involution forms.

In view of the varied morphological appearance, this has been used as basis of several classifications. But as the morphology of a given strain is by no means constant and may be modified by alterations in cultural conditions, these systems of classification are of doubtful value.

Cultural Characteristics. Growth of *C. diphtheriæ* occurs on the ordinary media, but it is more profuse on enriched media. One of the most satisfactory media in use is Loeffler's serum, which is prepared by adding 1 part of 1 per cent. glucose broth to 3 parts of sterile ox-serum and inspissating. The optimum temperature is 37° C., range 14°–40° C., and the optimum reaction is pH 7·2. The diphtheria bacillus is a facultative anaerobe, but only scanty growth is obtained in the absence of free oxygen.

In *fluid media*, such as broth, a moderate turbidity develops, and frequently a well-marked pellicle forms. The appearance, however, varies with the type of the organism.

On *solid media*, such as Loeffler's serum, growth is profuse; after 24 hours' incubation the colonies are small, 1 mm. in diameter, smooth, circular, opaque, greyish-white or creamy with a granular structure and easily emulsifiable. On further incubation the colonies become more irregular, and there is a definite differentiation into raised central and flatter peripheral portions.

Growth is not arrested by dilutions of potassium tellurite inhibiting many other organisms, and, in the presence of this salt, the colonies acquire a greyish-black appearance. This substance has consequently been added to various enriched media and useful selective media have been obtained. It is important to note that the characteristic morphological appearance may not be found when growth occurs on some tellurite media. A striking development has recently resulted from the use of such media.

McLeod and his co-workers (1931) employed a special medium containing fresh rabbit blood, special broth, potassium tellurite (0·04 per cent.) and agar, which they termed "chocolate tellurite agar". They found, on examination of swabs from cases of diphtheria, that the diphtheria bacilli presented different colonial appearances and they were able, on this criterion, to classify the majority into two separate and distinct sub-groups, which were termed *gravis* and *mitis*; later a third group, *intermedius*, was recognized. A few atypical strains were also found. These observations have been confirmed by a number of independent workers and many varieties of tellurite media are now in use (see

p. 459). The following characteristics of the various types have been determined; these tend to remain constant, but irregularities and variations have occasionally been recorded.

Gravis strains form grey or greyish-black, granular, striated colonies presenting a "daisy-head" appearance; ferment dextrin, starch and glycogen; produce a surface growth and granular deposit in broth; usually non-hæmolytic; morphology, as determined by observation of an 18-hour culture on Loeffler serum, lightly stained protoplasm with one or two dark areas, granules are seldom found; usually isolated from severe cases of diphtheria.

Intermedius strains give small, discrete, flat, black, granular colonies with a central papilla and slightly irregular edge; there is no fermentation of starch and glycogen, while with dextrin the results are variable; produce in broth a fine granular deposit with a clear supernatant fluid and no surface growth; non-hæmolytic; morphology (Loeffler's serum), predominance of barred forms; granules usually scanty but may be well-marked; frequently associated with a severe clinical condition.

Mitis strains form convex, smooth, black, shining, entire colonies; there is no fermentation of starch and glycogen, while dextrin gives irregular results; in broth there is a diffuse turbidity with a non-granular deposit and rarely a soft surface growth; usually hæmolytic; morphology (Loeffler's serum), classical irregular forms with granules; usually found in the milder types of diphtheria.

The clinical significance of these types is considered later.

Resistance. The diphtheria bacillus is readily destroyed by most bactericidal agents, such as heat at 56° C. which kills the organisms in 20 minutes. The resistance to potassium tellurite has been discussed. Drying and low temperatures are withstood for long periods.

Biochemical Activity. *C. diphtheriæ* exhibits little proteolytic activity. Growth takes place on gelatin without liquefaction of the medium; indole is not produced. Several carbohydrates are fermented with the production of acid only. In these tests Hiss's serum-water medium is employed and important sugars are glucose, maltose and saccharose; the two former are fermented, but there is never any fermentation of saccharose. This is a point of differential importance, as many, but not all, diphtheroids ferment saccharose. In typing the organisms, starch, dextrin and glycogen are of great value. Litmus-milk is not changed.

Serology. The most important antibody evoked by the diphtheria bacillus is the antitoxin. All strains produce the same toxin, which is neutralized by the antitoxin prepared against the toxin of one strain. The toxin is, however, distinct from that produced by the Preisz-Nocard bacillus.

Attempts have been made to sub-group strains of the diphtheria bacillus by means of the agglutination test; the results have, however, been irregular. Recent investigations, however, indicate that the three sub-groups, *gravis*, *mitis* and *intermedius*, are not antigenically related, and that each group contains a number of strains which are serologically distinct.

Toxin Production. The main offensive weapon of the diphtheria bacillus is the toxin. It is important to note that there exist strains possessing all the properties of the classical diphtheria bacillus, except that they do not produce an active toxin; these non-toxigenic strains are usually considered to be avirulent. The mutation of virulent to avirulent strains doubtless takes place, but a completely avirulent strain rarely, if ever, becomes virulent. Avirulent strains are consequently considered to be of little significance in the epidemiology of diphtheria. It is important to note that *gravis* and *intermedius* strains are nearly always virulent, whereas *mitis* strains are frequently avirulent.

Loeffler in his original investigations came to the conclusion, from the results of his animal inoculation tests, that the lesions were due to a bacterium acting locally and to some product produced locally and passing into the general system. Although definite lesions occurred in various regions, the diphtheria bacilli were only isolated at the site of inoculation. A few years later Roux and Yersin (1888–90) found that the injection of the sterile filtrate, after passage of the culture through a bacterial filter, into the guinea-pig produced the same lesions as the inoculation of the whole culture. In this way the production of an "exotoxin" by the organism was established. An antitoxin was later prepared and this neutralized the toxin. Confirmation of these results was soon forthcoming, and the introduction of antitoxin therapy was the natural sequence.

Although toxin production is a characteristic property of virulent diphtheria bacilli, the amount formed by various strains varies to a marked degree. The capacity to produce toxin does not appear to be definitely related to virulence, as excellent toxin-producing strains have been isolated from mild cases of diphtheria, *e.g.*, the Park 8 strain, a celebrated toxin-producer employed in most laboratories for the preparation of antitoxin, was originally isolated from a mild case.

The production of toxin also depends to a large extent on the cultural conditions, particularly the composition of the medium. The presence of peptone is essential, but the different makes of peptone vary greatly in their toxin-inducing value; those derived from the digestion of fibrin, such as Witte's or Difco, or from autodigestion of the meat, should be selected. Acid is detrimental, the reaction should therefore be definitely alkaline, *p*H 7·6–8·2. Hartley's broth (*q.v.*) is a very satisfactory medium and is extensively employed in the production of toxin. The medium should not be over-heated and, in sterilization, filtration with, or without, autoclaving at a relatively low temperature for a short time, is the common procedure.

Abundance of free oxygen is essential and, to obtain this, the medium is arranged in shallow layers with a large surface area in amounts of 2–5 litres in special flasks or cylinders, which are loosely plugged to permit free diffusion of air. The inoculum is the pellicle of a growth obtained in a "starter-bottle", which contains about 100 c.c. of the same medium. The diphtheria bacillus is acclimatized by passing through two or three starter-bottles before it is used for the preparation of toxin. This ensures good pellicle formation, which is necessary to obtain the maximum toxin production; this of course does not apply to the *intermedius* strains which do not form a pellicle. After inoculation or seeding, the flasks are incubated at 37° C. for 7–11 days. At the end of this time the broth is filtered and the filtrate contains the crude toxin, *i.e.*, toxin plus various products of digestion and disintegration of the components of the medium.

When freshly collected the toxin is relatively unstable; it is destroyed or modified readily by heat at 60° C., acids, alkalies, light and oxidation. By storage in the dark at a low temperature, 0°–4° C., it becomes more stable. This process, which is termed "ripening", is adopted when the toxin is required for testing purposes.

Toxin has been concentrated by various methods, such as salting out with ammonium sulphate or precipitation with acetic acid. It may be also obtained in a dried form and is then relatively stable. Its exact chemical composition has never been determined, as it is impossible entirely to separate it from the proteins of the medium.

The potency of the toxin is standardized in the guinea-pig

Varying dilutions are injected subcutaneously, and the smallest amount killing a 250-gm. animal in 4 days is termed the *minimal lethal dose*, or M.L.D. Another method is to determine the *minimal reacting dose*, or M.R.D., which is the smallest amount producing a definite reaction on intradermal inoculation into a guinea-pig. A good toxin is one having a M.L.D. of 0·002 c.c.

Toxoid or Anatoxine. The toxicity of a filtrate may be reduced by the addition of 0·2–0·4 per cent. formalin and subsequent incubation at 37° C. for 4–6 weeks. With the reduction in toxicity there is, however, no loss of antigenicity or combining power. The modified toxin, which is termed *toxoid* or *anatoxine*, is consequently a valuable immunizing agent. In its preparation the loss of toxicity is controlled by the subcutaneous injection of guinea-pigs, in which 5 c.c. of the toxoid should not give a reaction. The toxicity decreases rapidly at first, during incubation at 37° C., but it may take several weeks for its complete removal.

Toxin or toxoid is chiefly required for the immunization of horses in the preparation of antitoxin and for human prophylaxis. Owing to the severe reactions and the potential dangers following the use of toxin, it has now been largely replaced by the toxoid.

Pathogenicity. The pathogenicity of the diphtheria bacillus is largely dependent on its capacity to produce the powerful exotoxin. In the case of certain strains, however, there appears to be little doubt that other factors may be concerned. The same antitoxin neutralizes the toxins produced by *mitis*, *intermedius* and *gravis* strains, and yet serum-therapy in *gravis* and *intermedius* infections in man may have little effect on the course of the condition. The nature of the other pathogenic factors has not been determined, but they appear to be distinct from the toxin and may be intimately associated with the bacterial protoplasm.

Laboratory animals vary considerably in their reaction to the diphtheria bacillus according to the susceptibility of their tissues to the toxin. Rats and mice possess a high degree of resistance; this appears to be an example of natural species immunity, as the specific antitoxin is not present in the serum. Dogs and cats are moderately susceptible, while rabbits and guinea-pigs are highly susceptible. The guinea-pig, owing to its convenient size, is the laboratory animal chiefly employed in the study of this organism.

On subcutaneous or intramuscular inoculation of the guinea-pig with a small amount (0·5–1 c.c.) of a virulent culture, death usually follows in 24–72 hours. On autopsy an œdematous, slightly hæmorrhagic, local lesion is found with enlarged regional glands, congestion of the lungs and spleen, pleural exudate and marked congestion of the suprarenals. The congestion of the suprarenals is pathognomonic, being present in at least 80 per cent. of animals.

On intradermal inoculation a well-marked local lesion appears in 24 hours, becoming, in 48 hours, an erythematous swelling with a black necrotic centre.

In man the diphtheria bacillus is responsible for the spontaneous disease, diphtheria.

Diphtheria

Diphtheria is a common and widespread disease, which has been the subject of numerous and extensive epidemiological surveys. It is interesting to note that its main epidemiological features were recognized and that general prophylactic measures had been introduced long before the isolation of the causative organism had been accomplished.

Diphtheria is particularly a disease of children; some 85 per cent. of cases in this country occur in children under 15 years. The incidence is highest in the age-group 5–10 years, *i.e.*, the age-group when children usually commence school, but after the tenth year there is a sharp decline. It is a disease of schools and institutions, where groups of susceptibles are herded together. In such centres extensive outbreaks may occur in the absence of specific immunization.

Infection is conveyed from one individual to another either by direct contact or indirectly by droplet infection, and as a rule the lesions develop primarily in the naso-pharynx. The diphtheria bacillus produces a more or less characteristic reaction, which results in the formation of the so-called "pseudo-membrane." This is mainly a fibrinous exudate with disintegrating epithelial cells, leucocytes, red blood corpuscles and bacteria. It appears as greyish-white or yellowish patches usually on the tonsils, uvula, palate, larynx or trachea, and is frequently difficult to detach. The neighbouring mucous membrane is extremely congested and the regional lymph glands are usually swollen and painful.

General symptoms are also present due to the absorption into the circulation of the toxin produced by the organisms at the local lesion. The organs most frequently involved are the heart and the nervous system. Cardiac failure is not an uncommon complication of diphtheria, but the exact site of damage has not been determined. The lesion of the nervous system is a peripheral neuritis giving rise to paralysis involving mainly the palate, the limbs and accommodation of the eyes.

The suprarenals, in fatal cases, rarely show the gross macroscopical changes seen in the guinea-pig, but microscopical changes, such as infiltration and minute hæmorrhages, may occur.

Diphtheria is essentially a toxæmia, the organisms have only rarely been isolated from the blood-stream during life. They tend to remain at the primary focus of infection, and there produce the toxin which is responsible for many of the clinical features. The mortality rate is subject to much variation, but there is no doubt that it has been reduced considerably by the introduction of antitoxin therapy; in the pre-antitoxin period the average mortality was about 30 per cent., whereas it is now approximately 10 per cent. Death is frequently due to delayed administration of serum and may result either from respiratory obstruction by the membrane, as seen usually in the laryngeal form, or from toxæmia. Recovery confers a high degree of immunity and is associated with the presence of antitoxin in the serum; second attacks are extremely rare.

The severity of the condition is definitely related to the character of the infecting organism; *gravis* and *intermedius* strains are more frequently associated with severe forms of the disease than *mitis* strains. This variation in severity is well illustrated in an analysis by Wright of 8,040 cases of diphtheria occurring in the Liverpool district during the period 1937–40. With all types the mortality rate was highest in the age-group 0–4 years (Table XXII.).

TABLE XXII

Correlation of the Type of C. diphtheriæ with the Mortality Rate

Strain	Number of cases	Mortality rate per cent.
Gravis	4,126	6·3
Intermedius	1,518	10·7
Mitis	2,395	2·2

Diphtheria is widely distributed in the civilized world, but infection with the diphtheria bacillus does not by any means invariably produce the classical features of the disease. Lesions may be produced in the nose only, as a fibrinous rhinitis, in the ear or on the conjunctiva. In the naso-pharynx a membrane is not always produced and atypical forms may occur. Epidemiological and statistical studies indicate that sub-clinical infection is a frequent event; many adults possess a high degree of resistance, and yet they give no history of previous attacks of clinical diphtheria or immunization.

From all cases CARRIERS may result; *gravis* infections tend to be the most persistent while *intermedius* infections clear up most quickly. The diphtheria bacilli are present in the throat and/or nose during convalescence, and in some they may persist after the main lesions have resolved. Healthy individuals possessing increased resistance are frequent carriers of virulent diphtheria bacilli. Endemic foci are largely maintained by carriers, and there can therefore be no marked reduction in the incidence of the disease until prophylactic immunization becomes general. It is considered that some 30 per cent. of pre-school infants and 50 per cent. of school children must be immunized before any appreciable decrease in the incidence of diphtheria will be observed.

Diagnosis. It is important to note that the diagnosis of diphtheria is essentially the duty of the clinician, and on no account should it be considered the task of the bacteriologist. Whenever the symptoms are suggestive, the case should be treated as one of diphtheria; the bacteriological examination is employed to confirm the diagnosis. The isolation of the diphtheria bacillus does not necessarily indicate the case to be one of diphtheria and the failure to detect it does not exclude the disease. With these reservations there is little doubt that the bacteriological examination is of great value to both the clinician and public health official in dealing with cases and carriers.

Material for the bacteriological examination is collected on a sterile swab, which should be rubbed along the membrane and not pushed blindly into the mouth. The methods of examination are by direct microscopy or by culture and subsequent microscopy.

The direct slide method is sometimes useful in frank cases, but it is not recommended for routine use; the swab is rubbed first on a sterile slide, which is stained and examined, and then on to a Loeffler's serum slope. If positive an early report can be made, but an immediate negative result is valueless.

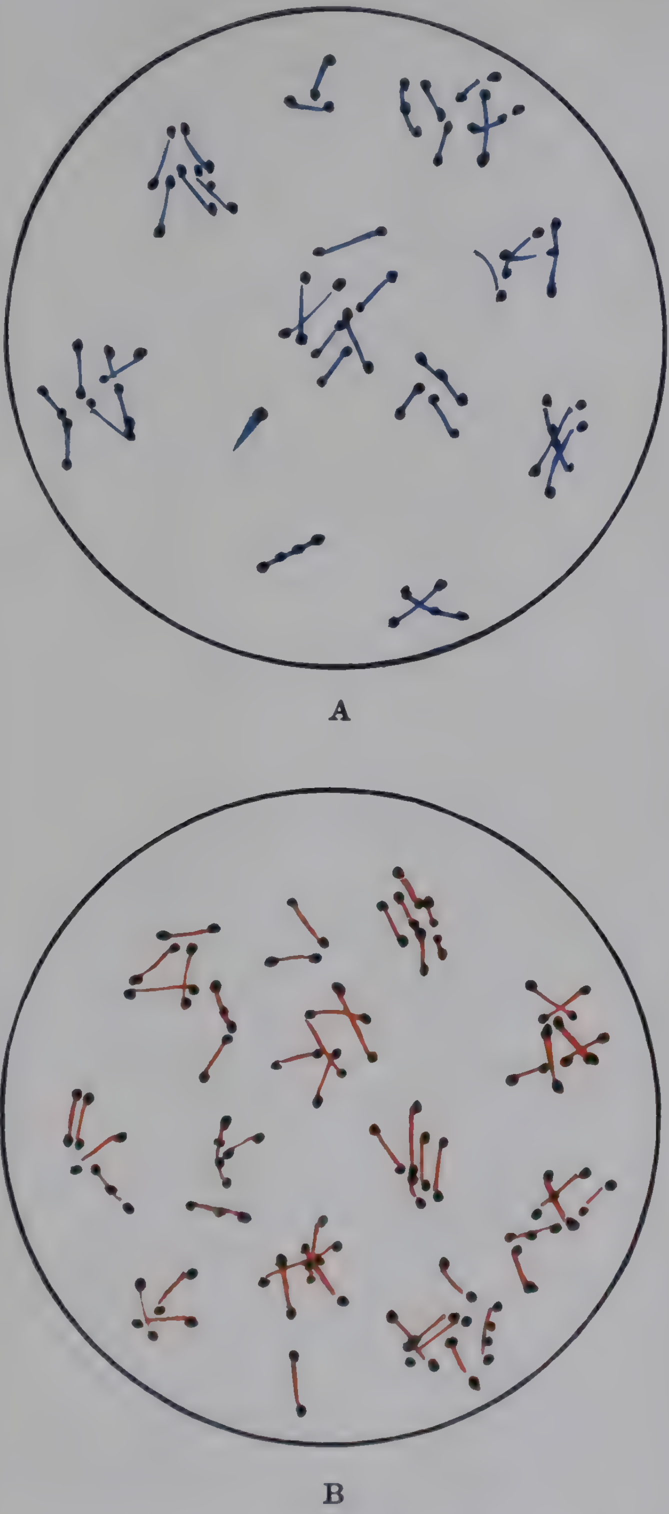
The usual method is to prepare cultures, and after incubation at 37° C. for 18–24 hours to carry out the microscopical examination. The standard medium is Loeffler's serum prepared as a slope, but now tellurite media are required and many different types are available, all giving satisfactory results. Each swab is rubbed on both a Loeffler's serum slope and the tellurite medium; after incubation overnight smears are prepared from the Loeffler serum culture, while the growth, if any, on the tellurite medium is examined through the hand lens. This requires considerable experience, as other organisms tend to produce blackish colonies, but with practice the typical colonial appearance of the various types of diphtheria bacilli can be detected. The results of the two examinations are considered together; in the great majority of cases there is complete agreement, but in some the microscopical examination may be negative while the tellurite reading is positive. In such cases sub-cultures are prepared from the tellurite on to Loeffler's serum, which is examined after incubation overnight at 37° C. Microscopy of the growth on the tellurite medium may be unsatisfactory. In some cases it is thus impossible to complete the examination in 24 hours.

In identifying the diphtheria bacillus by microscopical methods, great care must be taken to differentiate it from the diphtheroids, particularly *C. hofmanni*, which are frequently present in the naso-pharynx. The main diagnostic features of *C. diphtheriæ* are the pleomorphism, irregular staining with the presence of granules, and the characteristic arrangement (Plate II.). It is, however, important to note that granules are frequently absent from *gravis* strains.

C. hofmanni is short and relatively stout, does not usually possess granules, tends to be regular (pleomorphism is uncommon) and to be arranged in parallel rows; its protoplasm stains unevenly, presenting a " barred " appearance, due to the weakly stained central protoplasm. It also grows rather more readily on the simple media, such as agar, than the diphtheria bacillus.

In the search for carriers it is necessary to obtain swabs from both the throat and nose; for this purpose the use of plates containing a tellurite medium is essential. After incubation overnight a mixed growth is almost invariably obtained, and suspicious blackish colonies are picked off on to Loeffler's serum slopes, which are incubated at 37° C. for a further 24 hours. The morphological appearance of the organism is examined, and

PLATE II



C. diphtheriæ (diphtheria bacillus), *mitis* type, × 1000, stained (A) with Loeffler's methylene-blue, and (B) by Neisser's method.

[*To face p.* 290.

those resembling the diphtheria bacillus are kept for further examination. It has previously been stated that all diphtheria bacilli do not produce a toxin and that the avirulent strains have no epidemiological significance. There is, however, no morphological difference between virulent and avirulent strains; it is generally accepted that *gravis* and *intermedius* strains are almost invariably virulent but *mitis* strains may be avirulent. It is therefore often necessary, particularly in dealing with carriers, to determine whether the isolated organism is virulent or avirulent. This is carried out by means of the virulence test.

In performing a *virulence test* pure cultures must be obtained; the action of these on glucose and saccharose in Hiss's serum-water is noted; if saccharose is fermented the strains are discarded, as this sugar is never fermented by the diphtheria bacillus. The virulence of the non-saccharose-fermenters is next examined in the guinea-pig by either subcutaneous or intradermal inoculation. The growth on Loeffler's serum is emulsified to give a suspension of slight opacity. In the subcutaneous method 0·5 c.c. of this suspension is injected subcutaneously into two guinea-pigs, one of which has previously been given antitoxin. The control animal should remain alive, whereas if the organism is virulent the test animal should die in 24–96 hours, and on autopsy present the characteristic appearances. When dealing with pure cultures control animals are not usually required.

In the intradermal method the injection is made into the skin of the shaved abdomen of two animals; one, the control, is given 500 units of antitoxin intraperitoneally the previous night, and the other, the test animal, may receive 125 units of antitoxin 4–6 hours after the injection of the bacterial suspension. Definite lesions should develop in 24–48 hours on the test animal, but not on the control, which is protected by the prior injection of the antitoxin. The small dose of antitoxin is given to the test animal when several suspensions are being tested; it does not interfere with the local lesions, but prevents the animal dying from toxæmia. The intradermal method is gaining favour, as it is more economical than the subcutaneous test; six or eight suspensions may be tested on one animal, whereas only one suspension can be used in the case of the subcutaneous method.

A further test was recently recommended by Manzulla for the rapid diagnosis of diphtheria. This is performed by applying a sterile swab soaked in 2 per cent. aqueous solution of potassium tellurite to the exudate in the throat. It was claimed that exudates caused only by *C. diphtheriæ* turn black at the site of application. This observation has not been generally confirmed. Lesions caused by organisms other than the diphtheria bacillus may be turned black by the tellurite ; however, the absence of blackening of the exudate is a strong indication that the lesion has not been caused by the diphtheria bacillus. It is important to note that this test should not replace the usual diagnostic procedures.

Prophylaxis. Specific prophylaxis in combating such a widespread infection as diphtheria is only of practical value provided that it is possible to differentiate susceptible individuals from those possessing resistance to infection. This was made possible in the case of diphtheria by the introduction of an intradermal test by Schick, which is now universally employed and is known as the *Schick test.*

In the *Schick test* a small amount of toxin, corresponding to 1/50 M.L.D., and contained in 0·2 c.c., is injected intradermally into the flexor surface of the forearm. In order to have a measure of combining power as well as toxicity, the *Schick test dose* should also be neutralized when added to 1/750th, but not to 1/1,250th, part of an international unit of antitoxin. Official standards for the preparation of the Schick toxin were laid down by the Permanent Commission on Biological Standardization of the League of Nations in 1931. As a control an equal amount of the same toxin heated at 70° C. for 5 minutes, is injected into the skin of the right forearm. Four types of reactions may be observed :—

(1) Negative, in which signs of reaction do not appear at either situation.

(2) Positive, in which the control arm shows nothing while the test arm manifests a definite local erythema in 24–48 hours, this is usually well marked by the fourth day, then it gradually fades and presents a brownish appearance with perhaps some slight desquamation.

(3) Pseudo-reaction, in which at both sites there is a definite reaction in 24 hours ; this subsides more rapidly than the true reaction, and by the fourth day there is little to be seen. This is actually a negative result, the lesion being of a non-specific nature.

(4) Combined, in which reactions (2) and (3) are both present. When read at the end of 24 hours little difference is detected between the test and control arms, but this gradually becomes

more apparent, and by the fourth day it is striking; a well-defined positive result is then apparent on the test arm.

Reactions (1) and (3) are thus negative and indicate resistance to infection. It has been found that a negative result indicates the presence of approximately 1/100–1/40 unit of antitoxin in the serum. It has also been established that the presence of such an amount of antitoxin in the serum is a decided but not an absolute preventive against an attack of diphtheria.

Reactions (2) and (4) are both positive and indicate susceptibility to infection. The test dose for this reaction is selected as it is the largest amount of toxin usable without producing a reaction in individuals who have sufficient antitoxin in their serum to give a relative immunity. In view of the occasional occurrence of *gravis* infections in Schick-negative individuals, the test-dose may have to be increased. It has been suggested that a Schick toxin with at least three times the toxicity of the official standard should be used.

In tbe absence of either artificial immunization or an attack of the disease, most children are Schick-positive. The number of positive reactors tends to decrease with advancing years due to immunity acquired by subclinical infection. At one time most adults were considered to be immune, but in surveys recently carried out on military communities, it has been found that some 30–60 per cent. were Schick-positive, *i.e.*, susceptible to infection. This relatively high rate of positive reactors is the probable result of a general improvement of living conditions in this country as a result of which chances of subclinical infection have been reduced.

Having detected susceptible people, several methods of inducing a relative immunity are available. The following preparations have been employed for this purpose:—

(1) Toxin-antitoxin mixtures (no longer used).
(2) Formol-toxoid. (F.T.)
(3) Toxoid-antitoxin Mixtures. (T.A.M.)
(4) Toxoid-antitoxin Floccules. (T.A.F.)
(5) Alum-precipitated-toxoid. (A.P.T.)

(1) Toxin-antitoxin mixtures were first used for the immunization of man in 1913 by von Behring, but the development and widespread application of this measure were due largely to the efforts of Park and Zingher in America during the last two decades. The preparation of the mixture, in which the toxin is

just under-neutralized, requires much care. It is constituted so that it contains in 1 c.c. 3 units of antitoxin and 3 L + doses of toxin; the *L + dose of toxin* is the smallest amount that when mixed with 1 unit of antitoxin produces death of a guinea-pig of 250 gm. in 4 days. Three doses of 1 c.c. were given every 7–10 days. The results were generally satisfactory, but unfortunately in some cases severe reactions occurred and in a few instances serious accidents resulted. These accidents arose from various causes: the development of the *Danysz Phenomenon*, due to the addition of the toxin to the antitoxin in separate portions so that excess of free toxin was present in the final mixtures; dissociation of the combination of toxin and antitoxin has also resulted from the freezing of the mixture, and the small amount of phenol, added as a preservative, becoming concentrated, inactivated the antitoxin. In view of these mishaps and the introduction of the toxoid, the crude toxin is now seldom, if ever, employed for the purpose of immunization of man.

(2) Formol-toxoid (F.T.) has been extensively employed by Ramon in France. It is readily prepared, but although the specific toxicity of the toxin is greatly reduced by the action of the formalin, it tends to produce a marked non-specific reaction in sensitive individuals. For this reason it is considered to be unsuitable for general application in individuals over the age of 10 years.

(3) With the toxoid-antitoxin mixture (T.A.M.) the risk of severe reactions is somewhat reduced. It is prepared so that 1 c.c. contains 0·1 c.c. of toxoid, of which 5 c.c. on subcutaneous inoculation into the guinea-pig does not give rise to symptoms, and 25–50 per cent. of the amount of antitoxin required to neutralize the unaltered toxin; three doses are usually given at intervals of 1 or 2 weeks.

(4) Toxoid-antitoxin floccules (T.A.F.) are composed of a saline suspension of the precipitate formed when equivalent amounts of toxin and antitoxin are mixed. With this preparation the risk of reaction is reduced to a minimum. It has a relatively high immunizing value and is recommended for adults.

(5) Alum-precipitated-toxoid (A.P.T.) is a turbid suspension containing the precipitate resulting from the addition of alum to the toxoid. It has been found that the yield of antitoxin following its use in the immunization of the horse is greater than that from the unaltered toxoid. The toxoid is liberated slowly from the precipitate and the antigenic stimulus provided thereby is

maintained for a longer period. A.P.T. is now widely used for the immunization of children. The original one-shot method has been given up and two doses of 0·2 and 0·5 ml. at 3–4 weeks' interval are now recommended for infants, with a third dose on reaching school-age.

The choice of preparation depends to some extent on the age of the individual. Immunization should not be carried out under the age of 1 year ; infants frequently possess some immunity and their tissues do not respond very well. The most suitable time is at the period 1–5 years. The tissues of children are not so reactive as those of adults, and therefore in immunizing infants the selection of prophylactic is not important. Adults, however, sometimes exhibit very severe and unpleasant reactions, which are of a non-specific nature, after the injection of the crude toxoid. It is therefore advisable in dealing with adolescents or adults to determine the reactivity of the tissues. This is carried out by means of a test introduced in Canada by Moloney and Frazer and now known as the *Moloney test*. In this 0·2 c.c. of a weak dilution, 1/100–1/200, of toxoid is injected intradermally : the appearance of a well-marked local erythema and induration in 48 hours indicates a high degree of sensitivity. These individuals should be immunized with due precaution. Toxoid-antitoxin-floccules (T.A.F.) are the most suitable reagent ; if toxoid only is available doses smaller than usual must be given. Some workers consider that, as it has been found that "Moloney-positive" reactors possess a considerable degree of immunity against diphtheria, further immunization is unnecessary. Underwood (1935), however, following an investigation of the Moloney test in Leeds, concluded that there was no justification for the assumption that strongly positive "Moloney reactors" are usually immune.

The results obtained by the use of these prophylactics have been highly satisfactory. Some 80–100 per cent. of individuals become Schick-negative after the administration of the requisite course. The incidence of diphtheria in immunized individuals has been greatly reduced, but not entirely eliminated. It is interesting to note that many of the reported cases in Schick-negative individuals have been *gravis* infections, and that in most of these the severity of the attack has been reduced.

In this country little immunization had been carried out prior to 1940, but as a result of intensive propaganda campaigns much progress has since been made. It is important that this procedure should be popularized as diphtheria is now a preventable disease.

The incidence of, and the mortality from, diphtheria in New York and Toronto have been greatly reduced by the mass immunization of children.

Passive immunization by the injection of antitoxin is now seldom used as the resulting immunity is of short duration, lasting only 2–3 weeks. It is used mainly as an emergency measure, particularly in hospitals and in dealing with delicate children. The usual dose is 1,000 units subcutaneously.

Therapy. The essential feature of the treatment of diphtheria is the early and adequate administration of ANTITOXIN. In all cases where the symptoms suggest diphtheria, serum must be given at once without awaiting the bacteriological report.

The antitoxin is produced by the immunization of the horse—usually with some toxoid preparation. Samples of blood are collected at intervals and the antitoxin content of the serum estimated. A reasonable titre, such as 500–600 units per cubic centimetre, may be obtained after immunization for 2–3 months. Blood is then collected in citrate solution, and from this the serum is prepared. The titration is carried out by comparing its action against a potent toxin with that of a standard antitoxin in the guinea-pig. The L + and L*r* doses are both employed for titration. In France the *Lf* dose is used, but owing to the possible event of irregularities this method is only employed as a preliminary process in this country. The antitoxin is contained in the pseudo-globulin of the serum and is associated mainly with the beta globulin fraction. The antitoxin can be readily separated from the remainder of the serum by salting out with ammonium sulphate, and is obtained in a concentrated form and free from much extraneous protein. A recent development has been the preparation of serum "refined" by enzyme activity, which removes unwanted protein fractions. Concentrated serum is therefore a highly satisfactory therapeutic agent and may contain up to 2,000–3,000 units per cubic centimetre.

In practice the dose and route of administration vary with the age and condition of the patient.

The intramuscular route, into the muscles of the thigh, is usually employed, but in severe cases where a rapid action is desired the serum should be given intravenously. In considering the dosage, it must be remembered that it is easy to give too little, but difficult to give too much. There is no generally accepted scheme of dosage, but as a working basis it may be taken that the older the patient and the severer the attack, the larger the dose. In dealing with mild cases the average dosage is 2,000–10,000 units ; in the severe forms it is 10,000–50,000 units. In very severe cases doses up to 250,000 units have been given.

Serum-therapy has produced very satisfactory results, the incidence of complications and the mortality rate have both been reduced to a marked degree. The mortality rate is, however, still relatively high in *gravis* and *intermedius* infections. In these cases the administration of antitoxin not infrequently has little effect on the course of the disease. The nature of the offensive weapon of these strains has not been determined, but as it appears to be concerned with the bacterial protoplasm an antibacterial serum has been prepared. This has been given together with the antitoxin, but the results are not encouraging.

Penicillin has given good results in the severe forms of the disease when given to supplement the action of the antitoxin, for which it must be clearly understood that penicillin is not a substitute. Attempts to clear carriers by the local administration of penicillin have not been very successful.

CHAPTER XXIV

MYCOBACTERIUM : TUBERCULOSIS : LEPROSY

THE main generic features of this group are : slender rods staining with difficulty, but when stained are acid-fast ; clubbed, swollen or cuneate forms and even filaments with branches may occur ; non-motile, non-sporing, aerobic and Gram-positive ; growth on media slow ; several species pathogenic to man and/or animals.

This genus contains a number of organisms, some of which are important pathogens while others are harmless saprophytes. The first member to be described was *My. lepræ*, or the leprosy bacillus, which was observed by Hansen (1874) in the epithelial cells of the characteristic nodules of leprosy, but it was not cultivated. Some time later, in 1882, Koch published the results of his brilliant investigations on the tubercle bacillus and its *rôle* in the ætiology of tuberculosis. This work was repeatedly confirmed, and Koch later demonstrated that the mammalian tubercle bacilli could be divided into two types—the human and the bovine. About the same time further types of the tubercle bacillus were isolated from birds and cold-blooded animals and were termed respectively the " avian " and the " cold-blooded " types.

Wells (1937), investigating an epidemic disease in voles which on post-mortem examination resembled in some respects both tuberculosis and rat-leprosy, isolated an acid-fast organism which proved to be closely related to the human and bovine types and appeared to be a new type of tubercle bacillus ; this is often referred to as the vole bacillus.

In 1895 Johne and Frothingham described another member, Johne's bacillus, which they observed in cases of an enteritis in cattle now known as Johne's disease. Stefansky (1903) described an organism, the rat-leprosy bacillus, which he had isolated in 1901 from rats during an investigation of an outbreak of plague in Odessa.

During the period 1885–1906 a number of saprophytic members were also isolated and described. These are of importance to medical bacteriologists mainly because they might under certain conditions be mistaken for the tubercle bacillus. The most important are : (1) the Smegma bacillus or *My. smegmatis* which is frequently present in the smegma of males and females ; (2) the butter bacillus or *My. butyricum* which was first isolated by Rabinowitsch in 1897 from butter ; (3) the timothy-grass bacillus or *My. phlei* which is found on timothy-grass and may be present in dust and fodder ; (4) the Mist bacillus or *My. stercussis* which has been isolated from the fæces of various animals.

The organism of importance to medical students is obviously the tubercle bacillus.

My. TUBERCULOSIS OR THE TUBERCLE BACILLUS

The tubercle bacillus is a strict parasite and is essentially pathogenic. There is a definite biological distribution of the various types ; the human and bovine strains produce tuberculosis in mammals, including man, the avian strains in birds and the cold-blooded strains in cold-blooded animals and fish.

Morphology. These organisms are usually straight or slightly curved rods with rounded ends and are arranged either singly or in small groups ; clubbed and swollen forms are seen in cultures ; branching is rare. The size is variable, the average dimensions are 0·3–0·5 × 1·2–4μ ; when isolated from the body-tissues they tend to be longer and thinner than when grown under artificial conditions. Granular or beaded forms are not uncommon. It has been suggested by several workers that the tubercle bacillus may assume a filterable form, but the work on which this view is based is unconvincing. Much has stated that in chronic lesions (*e.g.*, cold abscesses) non-acid-fast, granular forms are present ; these have been termed " Much's granules ". This observation has not been generally confirmed.

Tubercle bacilli stain with difficulty owing to the presence in the cell of fatty and alcoholic materials ; the ordinary staining methods are consequently of little value. They are Gram-positive, but the staining is not easy, and this method is not usually employed in the study of the acid-fast organisms. The organisms when stained are particularly resistant to the decolorizing action of acids and alcohol ; this is stated to be due to the presence of a higher alcohol termed " mykol ". By virtue of this property members of this genus are frequently referred to as " acid-fast " organisms.

Owing to the difficulty in staining the tubercle bacillus a special technique is required for routine purposes. That in general use is one developed independently by Ziehl and Neelsen and usually referred to as the Ziehl-Neelsen method. This consists in covering the film or smear with strong carbol-fuchsin and gently heating until steam rises; the heating is continued for 5 minutes. The film is washed well with water and 15–20 per cent. acid, usually H_2SO_4, is added; the film, if pus or exudate, turns yellow, but becomes pink again on washing; the process should be continued until only a faint pinkish tinge reappears (usually 2–15 minutes). The film is then again washed well in water and counterstained with a weak aqueous methylene-blue solution for ½–1 minute; after this it is washed and dried. On microscopical examination the tubercle bacilli appear as red rods, while other organisms, excluding acid-fast species, and the tissues are blue (see Plate III.).

Cultural Characteristics. Growth under artificial conditions varies considerably with the different types of tubercle bacilli. The cold-blooded types behave as the saprophytic members of this group and grow readily on ordinary media in 2–3 days. The human and bovine types, on the other hand, are extremely difficult to culture; special media are required, and even then growth is very slow. The avian type occupies a position mid-way between these extremes.

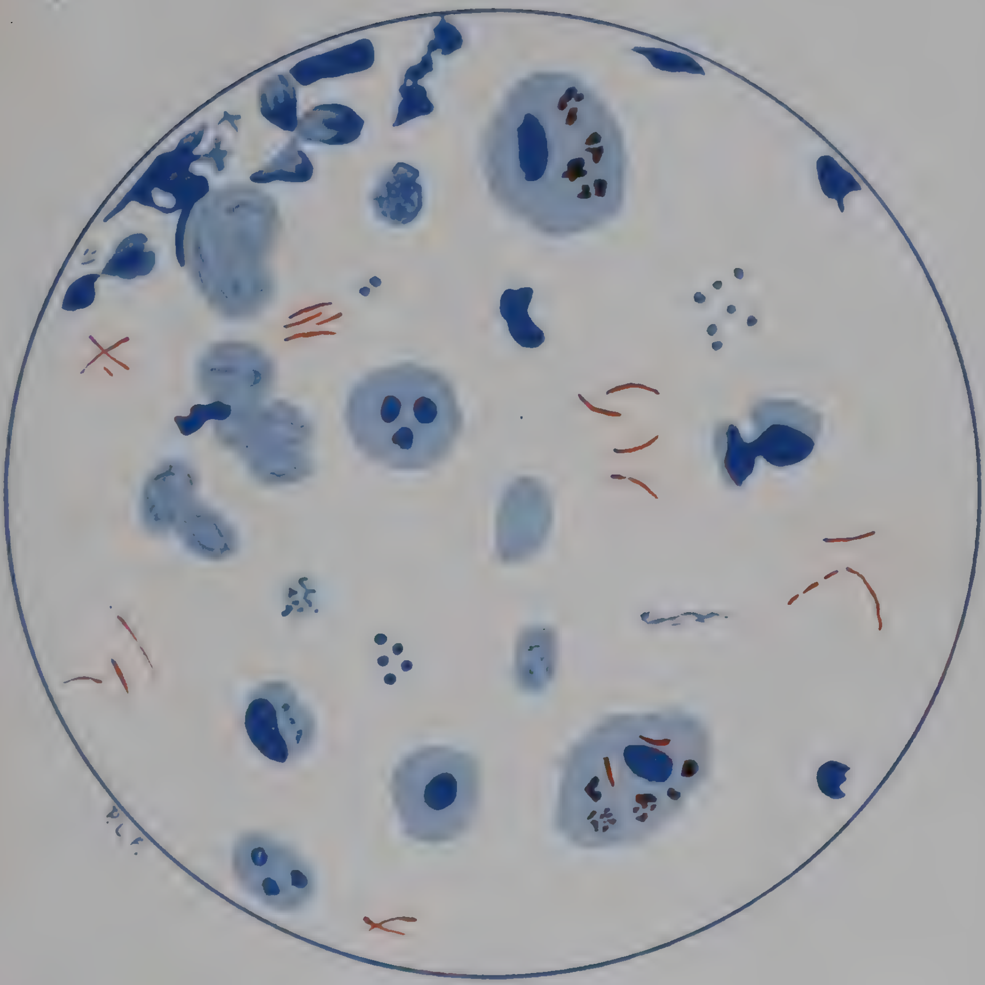
The media commonly used for studying the mammalian types are Dorset's egg, made by mixing eggs with water and inspissating at 70° C., inspissated serum, glycerine-agar or -broth and glycerine-potato. It is important to note that the addition of glycerine to agar or broth enhances the growth of all members of this group with the exception of the bovine strains of the tubercle bacillus.

Aerobic conditions and moisture are essential for growth, which is absent or very slight in the absence of free oxygen. The optimum reaction of the medium varies with the types, and is about pH 7·0–7·6. The optimum temperature also varies with the different types: for the human and bovine bacilli it is 37° C., for the avian about 40° C., and for the cold-blooded about 25° C.

On *solid media*, such as Dorset egg, growth occurs usually as dry, friable, confluent colonies with a granular or wrinkled surface. The bovine and human types grow slowly, 4–6 weeks being usually required to obtain an extensive growth, the plugs of the test-tubes or flasks must therefore be sealed with paraffin to prevent evaporation of the medium; it is more satisfactory to use bottles or tubes with screw caps.

In *fluid media*, such as glycerine broth, the human and bovine

PLATE III



Tubercle bacilli in sputum from pulmonary tuberculosis (stained by the Ziehl-Neelsen method). × 1000.

[*To face p.* 300.

types grow as a greyish-white, dull, wrinkled, surface pellicle with no general turbidity ; a slight granular deposit may be present. The avian and cold-blooded types show no surface growth or general turbidity, but give a granular deposit or a thick veil at the bottom of the flask. A peculiar fruity odour is given by cultures of all types.

The cultural reactions present features of importance in the differentiation of the human and bovine types.

The human type grows more freely, particularly when the medium contains glycerine, and for this reason it is termed *eugonic*. The bovine type usually grows less luxuriantly, particularly on fresh isolation on to glycerine media, when it may fail to grow, and is termed *dysgonic*. When growth is obtained on media containing serum of a rich yellow colour, the human type frequently forms a yellowish or orange pigment, the bovine type never does.

The cultural differentiation between the mammalian and the other types is not difficult. The avian type after a few subcultures usually grows readily at 40°–42° C., and in fluid media gives a granular deposit or a spreading growth at the bottom of the flask. The mammalian types form a surface pellicle in fluid media, and growth is slight or absent at 40° C. The cold-blooded types usually give an early and luxuriant growth when incubated at 25° C., at which temperature the other types do not multiply.

These four types appear to be stable. Attempts have been repeatedly made to demonstrate mutation of one type into another, but without success. Atypical forms have been isolated from a small percentage of lesions in man and animals. Some workers consider that these are types intermediate between human and bovine ; the majority of investigators, however, accept these forms as variants of one or other of the recognized types.

Resistance. The tubercle bacillus possesses an unusual property in that it behaves as other non-sporing organisms to the action of heat, but is very resistant to chemical bactericidal agents. In a dried state the organisms may survive exposure to 100° C. for 20 minutes, but as a saline suspension they are killed at 60° C. in less than 30 minutes. In milk the tubercle bacilli are killed by this treatment provided it is contained in a closed vessel. In an open vessel a surface pellicle forms ; this protects the organisms and renders their destruction more difficult.

Their resistance to chemical agents is similar to that exhibited by many sporing organisms and is doubtless due to the presence in the bacterial cell of fatty materials and alcohols, which resist the penetration of aqueous solutions. Marked resistance is shown to 5 per cent. phenol, 15 per cent. sulphuric acid, caustic soda and antiformin ; the two latter solutions are frequently employed in the isolation of the tubercle bacillus from contaminated material, as they digest the solid matter and destroy the Gram-negative bacteria. In expectorated sputum the organisms are rapidly killed if exposed to direct sunlight, but may survive for weeks if kept in the shade. The effect of the aniline dyes is variable ; the tubercle bacillus can grow in the presence of dilutions of gentian-violet and malachite-green that prevent growth of many organisms. These substances have consequently been employed in the preparation of selective media, *e.g.*, gentian-violet is added to coagulated egg in Petroff's medium, and malachite-green is employed in Lowenstein-Jensen's medium. The latter medium is now being used extensively, as growth occurs more rapidly than on the other varieties. It is composed of a starch solution, egg, mineral salts and malachite-green (p. 460).

Biochemical Activity. Little attention has been given to biochemical activities of the tubercle bacillus. Acid, but not gas, is produced in some sugars, *e.g.*, glucose and maltose, but these tests are seldom utilized in routine practice.

Serology. Agglutination, agglutinin-absorption and complement fixation have all been used in the study of the tubercle bacillus. Of these, however, only complement fixation is now employed as a diagnostic measure, and the results obtained are not as informative as could be wished. By means of these tests it has been found that the tubercle bacilli fall into three serological groups—mammalian, avian and cold-blooded. The human and bovine types constitute a homogeneous group and cannot be differentiated by serological methods.

Pathogenicity. The tubercle bacillus is one of the most widely distributed pathogenic organisms. It is essentially a pathogen and produces spontaneous tuberculosis in man and a large variety of animals. The susceptibility of the different animals varies, but cows, swine and monkeys are particularly sensitive. The various types of tubercle bacilli differ considerably in their pathogenicity. The mammalian types are pathogenic to man and mammalian animals, but not to birds or cold-blooded animals ; the avian type is pathogenic for birds, but has little action on mammals except rabbits and pigs ; the cold-blooded type produces tuberculosis in cold-blooded animals and fish.

Animals show marked differences in their susceptibility to experimental tuberculosis. Some species, *e.g.*, guinea-pigs and monkeys, are highly susceptible to both mammalian types; others, such as dogs, rats and mice, are very resistant. A third group, including goats, ferrets and rabbits, is relatively susceptible to the bovine bacillus but resistant to the human bacillus. This variation in susceptibility provides a further test for the differentiation of the two mammalian types; in practice the rabbit is the animal employed for this purpose.

The injection of tubercle bacilli into susceptible animals, such as the guinea-pig, produces a rapidly spreading fulminating form of tuberculosis ending fatally usually in 3–5 weeks. Following subcutaneous inoculation a well-marked local caseous lesion forms with caseating tubercles in the regional glands and most organs, particularly the spleen, liver and lungs. In relatively resistant animals, such as the rabbit for the human type, the subcutaneous inoculation of a small dose of the organisms produces a slowly developing local lesion; generalization of the infection and death may occur in some cases after several months.

Tuberculosis

Tuberculosis has been and still is one of the most important scourges of civilized communities. It appears to be one of the disadvantages of civilization, as the disease is extremely rare in uncivilized communities. When the tubercle bacilli have been introduced into these communities a fulminating type of tuberculosis, with early death, has resulted. In this respect it is interesting to note that the disease is also practically unknown in monkeys in their natural surroundings, but when these animals are brought into captivity tuberculosis becomes very common and usually runs a rapid course.

Tuberculosis in man may exist in a great diversity of forms and may involve practically every organ of the body. Its distribution and form depend largely on the age and susceptibility of the patient and the route of entry of the organism. In resistant individuals a localized, indurated, chronic lesion usually develops, whereas in susceptibles the acute generalized forms predominate. Between these two extremes a large variety of lesions is found.

Modes of Infection. In practically all cases infection takes place after birth. The old idea that infection might, in some cases, be hereditary is now largely discredited. Infants of tuberculous parents with active lesions frequently develop tuberculosis, but this is considered to be due to their greatly increased chance of infection after birth. The incidence of the disease in such infants has been decreased by their immediate removal to healthier surroundings. Tuberculous fœtuses have been removed from cows, but, while the infection might have occurred by the transplacental route, in many cases lesions have been demonstrated in the uterus and cotyledons of the parent.

In post-natal infection the tubercle bacilli may enter the body by any exposed surface. The three most important portals of entry are: (1) the respiratory tract, (2) the alimentary tract and (3) the skin. The infecting organism belongs to either the *human* or *bovine* type. Human infections with the avian type have been reported, but they are extremely rare.

(1) The Respiratory Tract. The pathogenesis of pulmonary tuberculosis is an unsettled problem. Two main hypotheses have been formulated: one, advocated strongly by English bacteriologists, postulates that infection occurs primarily by the way of the respiratory tract; the other, advocated by French workers, maintains that infection results mainly from passage of the tubercle bacilli through the intestinal mucosa.

In the inhalation theory the tubercle bacilli are introduced into the lungs either by droplet infection, *e.g.*, sneezing and coughing by patients with open tuberculous lesions, or in dust and dried sputum. Evidence supporting this theory is that respiratory tuberculosis is caused almost entirely by the human type, whereas the bovine type is largely responsible for abdominal tuberculosis. If infection occurred *viâ* the intestinal tract, one would expect the bovine type to be present in a greater number of respiratory lesions. The bacilli also pass through the mucosa of the upper respiratory tract and produce enlargement of the cervical or bronchial glands, from which they may spread to the lung.

The other hypothesis is based mainly on the observations of tuberculosis in animals. It is considered that, after absorption through the intestinal mucosa, the tubercle bacilli enter the lymph- and blood-streams and later pass to the lungs. The relative frequency of pulmonary tuberculosis in adults is explained by the reduced resistance of their lymph glands, as a result of which the bacilli are more liable to pass through and on to the lungs. This hypothesis appears less satisfactory than the former.

(2) The Alimentary Tract. Tubercle bacilli readily pass through the intact mucosa of the alimentary tract and reach the lymphatic system. The chief sites of entry are the mouth and naso-pharynx, giving rise to involvement and enlargement of the cervical glands, and the small intestine, particularly Peyer's patches, from which infection passes on to the mesenteric glands. The common vehicle of infection is milk; other sources include food and sputum.

(3) **The Skin.** Infection *viâ* the skin is relatively infrequent; it may occur either by way of minute abrasions during the handling of contaminated material and infected food or by direct inoculation, as by a contaminated knife or needle. Primary tuberculosis of the skin usually takes one of two forms, *lupus vulgaris* or *verruca tuberculosa*.

Histo-pathology. In all cases of tuberculosis the primary lesion, the *tubercle*, is essentially the same. This is a local lesion containing giant-cells, lymphocytes and epithelioid cells; the initial step in its development is the formation of microscopical tubercles, which increase and coalesce to produce the characteristic macroscopic tubercle. Further developments depend on the virulence and number of the organisms and the resistance of the host. Under conditions favourable to the host, the progress of the tubercle is arrested and fibrosis with perhaps ultimately calcification may occur. In this manner the focus of infection becomes either quiescent or healed. If quiescent, it may become active at some later date when the resistance of the individual is lowered.

Under less favourable conditions the lesion progresses. This sequence of events is therefore commonly encountered in individuals in whom the general resistance has been lowered by prolonged fatigue, under-nourishment, the acute exanthemata, such as measles and whooping-cough, injury and chronic alcoholism. In certain industries, such as those involving the treatment of silica and in miners (except coal miners), the inhalation of granules and the production of pulmonary lesions definitely predispose to infection with the tubercle bacilli. Under such unfavourable conditions the tubercle increases in size, degenerates, caseates and may form a cavity, ulcer or sinus. General manifestations of the infection, such as sweating and pyrexia, are then observed and result from the absorption into the general circulation of toxic substances produced by the tubercle bacilli. Extension of infection may take place by three routes: (*a*) directly along continuous surfaces, (*b*) by the lymph-stream and (*c*) by the blood-stream.

(*a*) Spread along continuous surfaces is frequently seen in ulcerative pulmonary tuberculosis. The breaking-down of a lesion in the lung or bronchial gland may result by aspiration in a tuberculous broncho-pneumonia and by expectoration in tuberculous lesions of the upper respiratory tract and face. In a similar manner tuberculosis of the epididymis may give rise to an ascending infection of the prostate and bladder.

(*b*) Spread by the lymphatics to the regional lymph glands is an early feature and occurs very commonly in children. There may be no lesion at the primary site of entry. In many cases the course of the disease is arrested at this stage, but in others there is a progressive involvement of other glandular groups and perhaps passage into the thoracic duct and then into the blood-stream.

(*c*) The entrance of the tubercle bacilli into the blood-stream is not uncommon ; it may occur not only by the thoracic duct, but also from any local lesion by invasion and ulceration of the wall of a vessel. When a few organisms enter, they tend to localize in various organs, particularly the bones, joints and kidneys, and produce a chronic or subacute type of lesion. If, however, a large number of organisms invade the blood-stream, there is a generalization of the infection with the production of miliary tuberculosis. In this condition small tubercles are present in many organs.

The Relation between the Type of Tubercle Bacillus and the Distribution of Lesions

It has already been stated that human tuberculosis is caused almost exclusively by the *human* and *bovine* types of the tubercle bacillus. As the *bovine* type gains entrance almost invariably by the intestinal tract and the *human* type usually by the respiratory tract, a different distribution of lesions is found. The types of tubercle bacilli in various lesions found in 1,321 cases in England and Wales have been collected by Griffith ; the main features are given in Table XXIII. Also there is a definite age-distribution of the different types of the disease ; as children are the usual milk-drinkers the *bovine* type of infection is more frequently found in children than adults (see Table XXIV).

These figures show that both types of tubercle bacilli are frequent causes of human tuberculosis ; the pulmonary form is, however, generally produced by the *human* type. The *bovine*

TABLE XXIII

Incidence of Bovine Tubercle Bacilli

Lesion	Total No.	0–5 Years		5–15 Years		Over 15 Years	
		No.	Per cent. *Bovine type*	No.	Per cent. *Bovine type*	No.	Per cent. *Bovine type*
Cervical glands	116	21	85·7	54	48·1	41	21·9
Lupus . .	176	59	61·0	99	51·5	18	16·6
Bone and joint .	511	84	28·6	342	19·0	85	4·7
Pulmonary .	202	0	—	10	0	192	1·0
Meningitis .	33	3	33·3	20	35·0	10	10·0

type is encountered mainly in children under 15 years of age and usually gives rise to non-pulmonary lesions. Griffith and Munro (1944) analyzed some 7,000 cases of pulmonary tuberculosis in Great Britain and found that approximately 6 per cent. of cases in Scotland were caused by the bovine type. The incidence was particularly high in the northern regions which are mainly rural with a flourishing cattle trade. Infection in such cases is conveyed by aerial transmission from other pulmonary cases of bovine origin or the open lesions of tuberculous cattle.

TABLE XXIV

Number of Deaths from Tuberculosis in 1929
(McNalty)

AGE GROUPS

Type of Infection	0–15 Years	15–45 Years	Over 45 Years
Respiratory . .	1,181	20,085	10,159
Non-respiratory .	3,524	2,119	922

These figures also indicate the serious problem presented by tuberculosis to the health of this country. The mortality rate has declined by 72 per cent. since the period 1851–60, but the disease is still responsible annually for some 37,000 deaths.

Immunity. The mechanism of the immunity reaction in tuberculosis is still unsolved. There is no doubt that some immunity may be acquired, but this is always of a low grade of effectiveness. Available data suggest that an increased resistance can only be produced by the living organisms and only in virtue of a previous infection is an immunity obtained. There is thus a condition known as an *infection-immunity*. Evidence supporting this view is obtained from a study of both the spontaneous human disease and the experimental condition in laboratory animals. Antibodies, particularly agglutinins, opsonins and complement-fixing bodies, have been demonstrated, but there appears to be no definite relationship between their presence in the serum of an individual and his resistance to infection with the tubercle bacillus.

Clinical tuberculosis in civilized communities is relatively uncommon among the adult population considering the possibilities of infection and, when present, it tends to run a chronic course. In infants and children the disease, on the other hand, is usually more acute. This suggests that adults are less susceptible to infection than children. On post-mortem examination it has been found that a large percentage of the adult population in an urban community has some localized focus of infection, such as an indurated gland. Adults also usually give a positive tuberculin reaction. These facts indicate that in virtue of the latent foci adults are endowed with an increased resistance to re-infection. On the introduction of tuberculosis into regions where the disease is uncommon, its course is the same in adults and children; an acute form of infection results. It is also recognized that non-specific factors, such as general health and good nourishment, play an important *rôle* in increasing the resistance to infection.

The immunity problem has been studied extensively in laboratory animals, particularly the guinea-pig, and it is from observations in these animals that much of our knowledge has been obtained. Koch (1891) found that after the subcutaneous injection of a small dose of tubercle bacilli into healthy guinea-pigs progressive lesions developed and the animals ultimately died. When, however, a similar injection was made into an animal, which had also been injected with a small dose of tubercle bacilli some 4 weeks previously, a slight lesion developed and cleared up rapidly; there was no involvement of the regional glands. This observation was confirmed and the reaction is now known as

" Koch's Phenomenon ". It was also found that if the second dose were given too soon after the first, *i.e.*, before a definite lesion had developed, the usual type of lesion developed. There was thus a marked difference in the behaviour of the healthy and the infected animals. When infected the guinea-pigs became both hypersensitive and more resistant to further infection. To this type of phenomenon the term " allergy " is now generally applied.

Allergy in Tuberculosis. Koch also found that the allergic reaction was manifested by guinea-pigs when, for the second inoculation, dead tubercle bacilli or extracts of the bacilli were employed instead of the living organisms. The bacterial extract was obtained by incubating tubercle bacilli in 5 per cent. glycerine broth at 38° C. for 6–8 weeks. After this it was evaporated in a water-bath at 100° C. to $\frac{1}{10}$th of its volume and passed through a filter. The filtrate was a brown, syrupy fluid containing about 40 per cent. glycerine, and was termed *tuberculin*. It is now known as Old Tuberculin. It is standardized by its action on guinea-pigs sensitized by previous infection with the tubercle bacillus.

The action of *tuberculin* was tested in the human subject, and it was found that healthy infants gave no reaction. In tuberculous patients, however, a very severe reaction was produced. The effect was thus analogous to that seen in guinea-pigs, in which the reaction has been studied in some detail. The reactions have been classified into : (1) local—formation of nodule with erythema, (2) focal—a short time after the second injection in the animals, it was observed that the tissue around the focus of infection becomes congested and œdematous, and (3) general—pyrexia, malaise, etc. The focal and general reactions are due to the absorption of tuberculin into the blood-stream. In view of the striking reactions given by tuberculous patients, tuberculin, administered by various routes, has been widely applied as a diagnostic measure.

The precise mechanism of the reaction has not been determined. There appears to be little doubt that, in the presence of tuberculosis, a local tuberculin reaction can be elicited in any of the body-tissues. It appears most probable that the reaction results from sensitization of the cells to some substance obtained from the tubercle bacilli and produced during the primary infection. It has also been shown that sensitization may follow the injection of killed tubercle bacilli. Some toxic substance is most probably produced by the interaction of tuberculin and the sensitive tissue-cells, and this gives rise to the general symptoms. The substance in the tuberculin responsible for the primary reaction is a protein; it is extremely heat-stable, but is destroyed by proteolytic enzymes. No significant difference has been detected in the tuberculins produced by the human and bovine types, but that formed by the avian type differs in several respects.

In tuberculosis the available evidence indicates that the allergic state is an expression of immunity. A negative tuberculin test indicates susceptibility or decreased resistance to infection, and is found most commonly in infants, members of uncivilized races and perhaps patients in an advanced state of tuberculosis.

Diagnosis. Many methods are employed in the bacteriological diagnosis of tuberculosis; the most important are: (1) microscopy, (2) culture, (3) pathogenicity, and (4) application of allergic reactions. Serological tests, particularly complement-fixation, have been employed, but the results are not always reliable. Non-specific tests, such as the sedimentation of blood corpuscles, have also been used; in the presence of an active infection there is generally a great increase in the sedimentation rate.

(1) **Microscopy.** The tubercle bacillus, owing to its characteristic staining properties, is one of the few organisms that may be identified by microscopical examination alone. The method in frequent use is that of Ziehl and Neelsen. Material from various sources may be submitted for examination. Sputum should be carefully examined and smears prepared from the thick nummular portions. When few organisms are present, the bacilli may be concentrated by centrifuging after treatment of the sputum with antiformin. As the smegma bacillus is frequently present, urine and fæces are usually decolorized with both acid and alcohol. It has been erroneously considered that this organism is not alcohol-fast, whereas the tubercle bacillus is; although this is not the case, the practice is still frequently employed as the tubercle bacillus usually tends to be more resistant to the action of alcohol than the smegma bacillus. Cerebrospinal fluid is either centrifuged or allowed to stand until a fine coagulum forms; this contains the bacilli and should be carefully teased out on a slide. In many cases decolorization of smears may be slow and repeated treatment with acid and alcohol is then required (see Plate III.).

The distribution of the tubercle bacilli in the focus of infection varies to a great extent according to the nature of the infection; in chronic lesions they may be scanty, while in acute spreading lesions they are most numerous.

(2) **Culture.** Cultural methods have not been generally used in the identification of the tubercle bacillus owing to its slow growth and the frequent presence of contaminating organisms in the material to be examined. Jensen, using a modified Lowenstein's medium (*q.v.*), has recently claimed striking successes by cultural methods. Contaminated material is mixed with 4 per cent. NaOH or 6 per cent. H_2SO_4; after standing 15–20 minutes it is centrifuged, and the sediment, rendered neutral to litmus, is seeded on to slopes of the medium. Growth of the tubercle bacilli readily occurs, and by 2–3 weeks it is claimed that the organisms may be typed. The human type gives large, rough, richly coloured orange-yellow colonies. The bovine colonies, on the other hand, are small, smooth, colourless and discrete.

(3) **Pathogenicity.** Most observers consider that the most delicate test for tubercle bacilli is the injection of the guinea-pig. Material is injected intramuscularly into the groin of one or two animals. Two animals give more satisfactory results than one, as one can be killed at the end of 3 weeks and the other after 5 weeks. In many, but not all, cases a positive report may be given in 3 weeks and valuable time is saved. By using two animals, the possibility of the test being nullified by death of the animal through intercurrent infection is also reduced. On post-mortem examination the distribution of the lesions varies considerably. When few organisms are present in the *inoculum* a local indurated or caseous lesion may only be produced; on the other hand, if the specimen contains many organisms, a generalized infection develops. Smears are prepared from the caseous material and stained by the Ziehl-Neelsen method.

These three methods involve the detection and/or isolation of the tubercle bacillus. In determining the type of the infecting organism the following criteria are of value.

Human tubercle bacilli are eugonic, *i.e.*, they grow relatively profusely on artificial media, particularly on those containing glycerine; they may produce a yellowish-orange pigment; they are of low virulence for rabbits as tested by the injection of 10 mgm. of the organisms subcutaneously.

Bovine bacilli are dysgonic, *i.e.*, they grow poorly on artificial media, the growth is not enhanced by the addition of glycerine to the medium, and they do not produce a pigment; they have a high virulence for rabbits.

(4) **Allergic Tests.** Koch's phenomenon has been widely applied in the diagnosis of tuberculosis in humans and animals. While

other preparations have been tried, Koch's old tuberculin is the substance mainly used in this test, which is frequently referred to as the "tuberculin test". In America a purified tuberculo-protein preparation has recently been used as a substitute.

A number of tuberculin tests have been employed at various times for the detection of tuberculosis. The best known are the subcutaneous method of Koch (1890), the cutaneous test of von Pirquet (1907), the conjunctival method of Calmette (1907), the intracutaneous test of Mantoux (1908), and the patch test of Vollmer (1937). Of these the tests of v. Pirquet and Mantoux have been most widely applied.

In carrying out the **von Pirquet test,** 2 drops of undiluted tuberculin are placed on the flexor aspect of the forearm about 4 in. apart; light scarification is carried out through the drops. As a control a similar scarification is made through 50 per cent. glycerine placed on a small area of skin between the drops of tuberculin. The tuberculin is allowed to soak in for 10 minutes. In a positive reaction, redness and swelling appear at the sites of the tuberculin inoculation in a few hours, and by 24 hours papules and perhaps vesicles may be present. The maximum effect is seen in 48 hours, after which it subsides. In a negative reaction slight traumatic effects are seen at the three points of scarification.

The **Mantoux test** is performed by the injection of 0·1 c.c. of a 1/10,000 dilution of old tuberculin intradermally into the forearm. If no reactions follow, increasing concentrations of tuberculin, *e.g.*, 1/1,000, 1/100, 1/10 and 1/1, are injected. The readings should be made after 2, 3 and 4 days, and in a positive result an area of erythema and induration, the diameter of which is at least 5 mm., appears at the site of the injection. A control is not required unless strengths of 1/10 or greater are employed; when used it consists of glycerinated veal-peptone-broth.

Hart (1931) carried out an extensive investigation of the tuberculin test. He concluded *inter alia* that the Mantoux test is more sensitive and less variable, being freer from technical error, than the v. Pirquet test; a positive tuberculin reaction indicates that the individual has at some time been infected by the tubercle bacillus, but does not distinguish between clinical and subclinical infection: in patients over 5 years of age the value of the positive test in the diagnosis of clinical tuberculosis is practically limited to observations made with the weak dilutions of tuberculin; a nega tive reaction excludes the presence of infection; tuberculin tests are of doubtful prognostic value. The Mantoux test is thus a valuable weapon in the campaign against tuberculosis.

Prophylaxis. Many measures have been advocated for the prophylaxis of tuberculosis. These have been directed both to the prevention of infection and to increasing the resistance of the community.

In preventing infection with bovine tubercle bacilli attention has been given to the condition of the milk consumed. By repeated inspection of the cows and bacteriological examinations of the milk this has been graded into different classes. Only those grades guaranteed free from tubercle bacilli should be consumed in an untreated condition. When contaminated the milk should either be pasteurized in bulk or boiled in the home. By the adoption of these measures the incidence of bovine tuberculosis can be greatly reduced, and it is particularly unfortunate that pasteurization is not being more generally employed.

The incidence of human tuberculosis has also been decreased by the segregation of open cases, and by drawing the attention of the public to the dangers of expectoration and coughing in crowded places such as trams, buses, etc.

General and specific methods have been used to increase the individual resistance. The general measures include attention to the social condition of the community, particularly their nourishment and general health, access to fresh air and sunshine, and the administration of cod-liver oil.

Specific Prophylaxis. *Tuberculin.* When Koch first introduced tuberculin he considered that he had a valuable therapeutic agent. The results were, however, extremely disappointing. Other preparations were later introduced: tuberculin O—a watery extract of tubercle bacilli disintegrated by grinding in an agate mortar; tuberculin R—the residue remaining after the removal of tuberculin O suspended in water, and tuberculin B.E.—a suspension of the organisms in 50 per cent. glycerine. These were tried for prophylactic purposes, but the experimental results were unsatisfactory.

The Bacille-Calmette-Guérin, or B.C.G., was introduced by Calmette and his co-workers. It is a bovine strain of tubercle bacillus that has been rendered avirulent by cultivation through many generations on a glycerine bile potato medium. The avirulence of this organism is considered by Calmette to be permanent and was established by injecting a number of susceptible animals, including guinea-pigs and monkeys, with large doses both subcutaneously and by the mouth. Calmette also claimed that after receiving 50 mgm. of B.C.G. subcutaneously a number of calves were unaffected by the subsequent intravenous injection of 5 mgm. of virulent tubercle bacilli, which dose was sufficient to kill untreated animals in 2 months. After prolonged experimental work on laboratory animals, Calmette used the B.C.G. for human prophylaxis. Infants born of tuberculous parents were at once removed and were also given B.C.G. by the mouth. It was found that the incidence of tuberculosis in these infants was much below the average. The application of the B.C.G. in human prophylaxis increased enormously on the Continent, but it has not yet been used on any scale in this country. While the relative innocuity of the organism is accepted, the use of the living bacilli does not readily commend itself. Lesions may be produced, but these are retrogressive. The degree of immunity following the use of B.C.G. appears to develop slowly and also to be of a low order. It is also well known that the incidence of tuberculosis can be reduced by the removal of infants from tuberculous parents, and this fact prevented a ready acceptance of Calmette's statistics.

Spahlinger has claimed outstanding successes both in prophylaxis and therapy by the use of vaccines, incomplete details of which have been published in recent years. The important point in their preparation appears to be that bovine strains must only be grown on media containing bovine tissue, such as blood or serum, and human strains only on media containing human tissue. These vaccines were secret preparations until recently, and while trials of their immunizing action in calves carried out in N. Ireland (1935) appear promising they are far from complete. A more serious and scientific investigation is required before the value of these vaccines can be assessed.

The Vole bacillus, while highly virulent for the vole, is relatively avirulent for rabbits and guinea-pigs. Experiments have consequently been carried out to test the value of this organism as an immunizing agent in guinea-pigs and the preliminary results have been very satisfactory. The subcutaneous inoculation of small doses of a pure culture of the vole bacillus afforded a high degree of protection against a later inoculation of virulent tubercle bacilli. These results are encouraging but it is essential that they should be repeated in other animals, such as the monkey, before an opinion can be given of the value of the vole bacillus as an immunizing agent against tuberculosis for general application.

Therapy. The original attempts of Koch at the therapy of tuberculosis by means of tuberculin were particularly disappointing in view of the hopes expressed. The doses employed were too large ; serious and even fatal reactions developed and the practice fell into disrepute. Other tuberculin preparations were introduced without any striking success. The use of minute doses in the treatment of chronic localized lesions has, however, given more satisfactory results. Many workers have consequently suggested that the use of tuberculin in minute doses should be more widely applied in the treatment of tuberculosis. The initial dose should be extremely minute (0·1 c.c. of a 1/1,000,000 dilution), subsequent doses may be gradually increased in the absence of a marked reaction ; great caution must, however, be exercised. In all instances specific therapy should be supplemented by general therapeutic measures.

Chemotherapy with arsenic and gold salts, *e.g.*, sanocrysin, has been tried without any striking success. The sulphonamides, in particular promanide, and penicillin have also given disappointing results. Preliminary trials suggest that the new antibiotic, *streptomycin*, might prove useful, but this work is still in the experimental stages and a definite opinion is not yet possible.

Leprosy

Leprosy was once a widely distributed disease, but it is now mainly restricted to endemic foci in countries where a low standard of living exists. It is chiefly encountered in Asia, particularly parts of China and India, Africa and South America.

Leprosy is a human disease of marked chronicity found mainly in children and young adults. It is apparently transmitted by direct and prolonged contact with cases, although the repeated failure to transmit the condition to healthy individuals by direct contact does not support this theory. Attempts to reproduce the condition in experimental animals have proved unsatisfactory, and it has therefore been necessary to study the pathogenesis of the disease by its course in man. The sources of infection are those cases with ulcerating nodules, skin abrasions and with organisms present in the saliva or nasal discharges. The main portals of entry of the leprosy bacilli are the naso-pharyngeal mucosa, the skin, the respiratory and alimentary tracts. After gaining entrance to the tissues the course of infection is obscure.

Clinical manifestations of infection may not appear for a considerable time, varying from a few weeks to many years. Lesions may be found in most tissues, but the nerves, the skin and the naso-pharyngeal mucosa are most frequently involved.

The essential lesion is a granuloma, containing connective tissue-cells, lymphocytes and large endothelial-like cells, which frequently contain acid-fast bacilli and have been termed " lepra " cells. In some cases the disease is extremely chronic and mild, but where the resistance is reduced the course becomes relatively subacute or acute and the lesions become more extensive. Disfigurement is a common feature. Two main clinical forms have been described—the nodular and the anæsthetic. This classification is, however, far from satisfactory and it is suggested that the types should be termed " lepromatous " and " neural ". While nodular or lepromatous lesions are usually found in the skin or mucous membranes and the anæsthetic or neural type is associated with lesions in the peripheral nerves, these forms frequently merge into each other. In some skin lesions nodules are not found and a diffuse or macular reaction is present. As a rule, when the nerves are involved, the disease runs a more chronic course than when the lesions are present in the skin. The course of infection appears to depend on the resistance offered by the tissues ; when this is marked and relatively few organisms are present the organisms tend to pass to the nerves. Inversely the more severe the lesion the more frequently is the skin involved. There is thus no clear line of demarcation between the various types of leprosy.

Leprosy, *per se*, is seldom fatal ; death frequently results from some intercurrent infection. Spontaneous regression may occur at any stage as a result of some increase in the general resistance of the body. Therapeutic measures involve attention to the general health and the administration of such drugs as chaulmoogra oil and potassium iodide.

Bacteriology. The leprosy bacillus was first described by Hansen in 1874, who saw large numbers in the granulation tissue-cells of leprous lesions. These observations have been repeatedly confirmed. The bacilli are usually situated in large numbers in the " lepra " cells and are often present on the naso-pharyngeal mucosa. They stain with difficulty ; they are straight or slightly curved, Gram-positive, acid-fast rods, $1{\cdot}5$–8μ in length, usually arranged in clusters. They are non-motile and non-sporing.

The organism has never been satisfactorily cultivated.

Numerous attempts on a great variety of media have been made. Successful results have been claimed, but conclusive evidence of multiplication of the organisms under artificial conditions has not been obtained. Under such conditions it is not unnatural that other organisms, such as other acid-fast bacilli and certain diphtheroids, have been incriminated as the ætiological agents of leprosy. Their relationship to the disease has, however, never been conclusively substantiated. Attempts to reproduce the disease in animals have also been unsatisfactory.

The clinical diagnosis may be confirmed by the demonstration of acid-fast bacilli, particularly inside the "lepra" cells, either in smears or sections stained by the Ziehl-Neelsen method. If doubt exists, tissue containing the organisms should be ground up and injected into guinea-pigs. The absence of lesions suggests the leprosy bacillus.

CHAPTER XXV

VIBRIO: CHOLERA: SPIRILLUM

THE main features of the genus *Vibrio* are: short, bent rods, sometimes almost straight; motile by means of a single polar flagellum (rarely two or three flagella are present); aerobes, growing well on ordinary media; frequently liquefy gelatin; do not form spores; Gram-negative; some species are pathogenic to man and animals.

The first member of this group to be described was *V. choleræ*, or the cholera vibrio, which was isolated from the fæces of cholera patients by Koch in 1884 during an epidemic of cholera in Egypt. Koch investigated the organism in detail and, as he was unable to isolate it from patients who had died of other diseases, he concluded that it was the causative agent of cholera. This view was not, however, accepted by all workers at that time, as the organism could not be isolated from the fæces of all cholera cases. This failure is probably explained by the inadequacy of the technique used and by the mild nature of the cases generally occurring at the end of an epidemic. V. Pettenkofer and a colleague were disbelievers and, to demonstrate their disregard for *V. choleræ*, they added the vibrios to their drinking-water. As a result Pettenkofer developed slight diarrhœa, while his colleague had a severe attack of cholera; from the stools of both *V. choleræ* was isolated and its pathogenicity became more appreciated.

Organisms resembling the cholera vibrio were also isolated from other sources, and this further confused the issue. Some were obtained from water and others from the fæces of individuals suffering from diarrhœa or intestinal conditions other than the true cholera; these were termed "para-cholera vibrios" by Kraus in 1909, and included the El Tor vibrio which is closely related to *V. choleræ*, the Finkler-Prior vibrio and many other varieties. The genus as a whole has not been studied in detail, and our knowledge is consequently far from complete; whether these organisms are all separate species or merely variants of *V. choleræ* has not been determined.

Vibrios are also responsible for diseases in birds and animals.

One was isolated by Gamaléia in 1888 from cases of enteritis in fowls and was designated *V. metchnikovi*. Another was obtained from the uterine discharge of sheep suffering from infectious abortion and also from the fœtuses of aborting cattle and was termed *V. fetus* by Smith (1918).

Many saprophytic members exist, and they have added confusion to the study of this genus. A number of vibrios have been isolated from water, which appears to be their natural habitat. Other sources include the nasal secretion, carious teeth, cheese, hay-infusion and manure.

Although owing to the incomplete state of our knowledge a general classification of the vibrios is impossible, *V. choleræ* has been subjected to extensive investigations, and its relationship to cholera is now universally accepted.

V. choleræ or the Cholera Vibrio

Morphology. The cholera vibrio, on first isolation from the tissues, tends to be in the form of a curved rod (0·3 × 1–5μ in size) and bears a close resemblance to a comma ; it has therefore been termed the " comma " bacillus. It may occur singly, in S-shaped pairs or spirals. After prolonged cultivation pleomorphism is marked and straight rods and involution forms are common. It is actively motile, due to the presence of a single terminal flagellum. It is non-sporing, non-capsulated and Gram-negative.

Cultural Characteristics. *V. choleræ* grows readily on the ordinary media. It prefers an alkaline medium, the optimum reaction being about *p*H 8. For isolation from contaminated material, *e.g.*, fæces, special selective media with a high *p*H, such as that introduced by Dieudonné, are therefore employed. The optimum temperature is 37° C., but growth occurs over a wide range, 16°–42° C. The cholera vibrio is a doubtful facultative anaerobe, growth being very slight in the absence of free oxygen. On potato a yellowish-brown pigment is formed by many strains.

On *solid media*, such as agar, after incubation for 24 hours at 37° C. there is an abundant growth ; the colonies are moist, translucent, greyish, round and small (1–2 mm.).

In *fluid media*, such as broth, multiplication rapidly takes place. After incubation for 24 hours there is a moderate turbidity with a thick surface pellicle and a slight deposit.

Resistance. *V. choleræ* behaves as most vegetative organisms and is readily destroyed by bactericidal agents ; heat at 55° C.

kills the vibrios in 15 minutes. The life of the cholera vibrio outside the body is short; varying from a few days to a few weeks according to the environmental and atmospheric conditions. This observation is based on several experiments carried out to determine the viability of the organism in fæces, fresh-water, sea-water, etc.

Biochemical Activity. *V. choleræ* has marked biochemical activity. This is seen particularly in its proteolytic actions which are most pronounced when the organism is grown in an alkaline medium. Indole is rapidly produced and nitrates are reduced to nitrites. These changes are produced simultaneously and form the basis of the "cholera-red" reaction, which was originally considered to be given only by *V. choleræ* and was used extensively for identification; it has since been found that the reaction is produced by many other vibrios. In carrying out this test a few drops of concentrated sulphuric or hydrochloric acid are added to a 24-hour peptone-water culture; a pink coloration results from the action of the acid on the indole and nitrites produced from the peptone by the cholera vibrio. Gelatin and coagulated serum are slowly liquefied; NH_3 and H_2S are formed.

The saccharolytic activity is also important; acid, but not gas, is formed from glucose, lævulose, maltose, mannite, saccharose and starch. Lactose is sometimes fermented late (5–12 days). In litmus-milk acid or acid and clot may be formed.

Observations on the production of a hæmolysin have been very discordant. As a general rule *V. choleræ* does not produce a soluble hæmolysin capable of lysing suspensions of red corpuscles. This property is, however, possessed by a few strains, such as El Tor, and by other vibrios. The results obtained by the use of the blood-plate technique have been most unreliable.

Serology. Serological reactions play an important *rôle* in the identification of the cholera vibrio. The tests commonly employed are agglutination and the bactericidal reaction known as "Pfeiffer's phenomenon".

In demonstrating Pfeiffer's phenomenon, the vibrios may be injected intraperitoneally into either an immune guinea-pig or a normal animal after mixture of the organisms with an immune serum. Lysis and disintegration of the organisms occur as a result of the action of complement on the sensitized vibrios. This action can also be demonstrated *in vitro*.

In the agglutination test several factors are concerned; being motile *V. choleræ* possesses both the heat-stable O or somatic component and the heat-labile H or flagellar component. As *V. choleræ* is monoflagellate the H factor is less pronounced than in the case of the Salmonella organisms. Gardner and Venkatraman have recently shown that the H factor is non-specific, being common to all strains of *V. choleræ* and the para-cholera vibrios, while the heat-stable O antigen contains two factors, one of which is specific for *V. choleræ* and the other non-specific.

The various phenomena associated with the smooth → rough (S→R) change may also be found. A complex polysaccharide, which behaves as a haptene, has been demonstrated and is considered to be associated with the somatic antigen, most probably the specific factor. The agglutination test shows a high degree of specificity and is now used extensively in diagnosis in place of the more complicated bactericidal reaction. There is, however, no doubt that the specificity of the agglutination tests will be considerably increased by the use of standard. sera prepared against the specific O antigen.

Pathogenicity. *V. choleræ* produces the spontaneous disease, cholera, only in man. It is pathogenic to most laboratory animals; guinea-pigs and rabbits have been usually used for experimental work. Following intraperitoneal inoculation a peritonitis rapidly develops, followed by septicæmia and death in 24–48 hours. Infection also results from the introduction of the vibrios *per os* after the gastric juice has been previously neutralized by the administration of bile.

There appears to be no doubt that a toxin is produced by *V. choleræ;* the profound collapse shown by cholera patients indicates very strongly the generalization of some toxic substance, but marked discrepancies are found in the reports of different workers on its formation and nature. The available evidence suggests that it possesses many of the properties associated with the so-called "endotoxins", and is liberated by the disintegration of the vibrios.

Cholera

Cholera is a widespread disease which is particularly prevalent in the East. In view of its geographical distribution it is frequently referred to as Asiatic cholera. The main endemic focus is India, especially the region around the delta of the Ganges. Epidemics have appeared in most countries and the spread of infection has invariably been along the trade routes and other links of communication. The ultimate source of the infection is the fæces of either cases or carriers. The vehicle of infection is in the majority of cases drinking-water, but milk, food, flies and direct contact, *i.e.*, contamination of the hands, have also been incriminated. The last extensive outbreak in this country was the Broad Street pump epidemic in 1854, in which infection was conveyed by contaminated well-water.

It is recognized that all individuals ingesting the cholera vibrio do not develop cholera. This individual variation does not appear to be due to any specific immunity but rather depends on the acidity and action of the gastric juices, which readily destroy the vibrios under normal conditions.

The onset of the condition usually commences some 24–48 hours after the ingestion of the cholera vibrios and is characterized by pyrexia, abdominal pain and diarrhœa. The symptoms rapidly become more severe ; a subnormal temperature, profuse diarrhœa with rice-water stools, suppression of urine, cramps and toxæmia are found. There is marked dehydration of the tissues. The infection usually remains localized in the intestines ; invasion of the blood-stream is extremely rare. The mortality rate varies from 30–80 per cent. Recovery is slow, but is not usually accompanied by complications. The vibrios are excreted in the fæces during convalescence by most cases, and in a few this excretion continues after recovery. These carriers do not, as a rule, excrete the vibrios for more than a few weeks. Healthy carriers, such as attendants of cases, may also occur but the excretion of the organisms by these individuals is of short duration.

In fatal cases the most important lesions are found in the lower half of the small intestine, the mucosa of which is congested, necrotic and desquamated. The intestinal contents are usually clear and fluid, containing a large number of vibrios and flakes of mucous membrane, which give the characteristic " rice-water " appearance. The vibrios are frequently present in the bile and gall-bladder, and a cholecystitis may be present.

Diagnosis. The bacteriological methods used in the diagnosis of cholera are (1) the isolation of the vibrio from the fæces or vomit, and (2) the detection of agglutinins or bactericidins in the blood-serum.

(1) When the vibrios are present in large numbers they can be readily recognized on microscopical examination ; their isolation is then relatively simple. When the numbers are small,

care is required. Satisfactory procedures are the use of special alkaline media, such as Dieudonné's, or serial cultivation in peptone-water (pH 8·4) with subculture from the surface growth after a short time, usually some 4–6 hours. The organism is finally identified by its biochemical and serological reactions. The isolation of *V. choleræ* from sporadic cases is sometimes a matter of considerable difficulty and requires much experience.

(2) The serological test now employed is agglutination, which has replaced the bactericidal test in most laboratories. Agglutinins appear early, but owing to the low titre their presence is usually of little diagnostic value before the sixth day. A titre of 1/100 or more is usually taken to be diagnostic.

Prophylaxis. The prophylactic measures adopted in the prevention of cholera are similar to those employed in dealing with enteric fever. Great care is taken at ports to prevent the entrance of cases or carriers into countries free from cholera. In all suspicious cases examinations should be performed for the presence of agglutinins in the serum and of the vibrios in the fæces. In infected regions strict attention should be paid to the food, drinking-water and general sanitary arrangements.

Many attempts at specific immunization have been made since Ferran used a living culture in 1885, but an entirely satisfactory vaccine has not been produced. Attenuated, phenolized, etherized and heat-killed cultures have all been tried. Bili-vaccines, given by the mouth, have also been used. The ordinary vaccine, prepared by heating a culture at 56° C. for 1 hour, has proved as satisfactory as the others. The immunizing substance is associated with the somatic and not the flagellar antigenic component. The disadvantages of vaccines are that reactions may follow their injection, and even after several doses the increased resistance is of short duration, 1–2 years, and further courses may be required. Statistics indicate, however, that vaccines have proved a useful prophylactic weapon. The usual dosage is 1,000 and 2,000 million vibrios at an interval of 7–10 days.

Therapy. Serum-therapy has been tried by many workers. The sera have been prepared by the injection of horses with the killed whole vibrios and also with the so-called " toxin ", but the results have been extremely disappointing.

Bacteriophage has been used as a therapeutic agent, but the results of its use are still incomplete.

Genus Spirillum

The generic characteristics of this group are: rigid, spiral rods with great variation in length, number and breadth of the spirals; usually motile by means of polar flagella; frequently Gram-positive; grow readily on simple media, some species producing a yellowish pigment.

These organisms have been frequently isolated from water and are practically all non-pathogenic to man or animals. They may, however, be mistaken for spirochætes or vibrios on morphological examination. One member, *Sp. minus*, is responsible for rat-bite fever, which is a febrile disease produced in man by the bite of an infected rat after an incubation period of several weeks. The organism was originally considered to be a spirochæte, but it has since been proved to belong to the *Spirillum* genus. It was first described by Japanese workers, but it has since been isolated in many countries, including Great Britain.

CHAPTER XXVI

PFEIFFERELLA: GLANDERS: MELIOIDOSIS

THE definition given by Buchanan to the *Pfeifferella* genus is: non-motile rods, slender, Gram-negative, without spores, staining poorly, sometimes forming threads and showing a tendency to branching. Gelatin may be slowly liquefied; do not ferment carbohydrates; growth on potato characteristically honey-like.

The name "Pfeifferella" was apparently adopted as a result of a clerical error. It was originally intended as the generic name for the influenza group of organisms, but, when "Hæmophilus" was considered to be more appropriate, "Pfeifferella" was erroneously substituted for the proposed name "Loefflerella" as the generic name of this group. In order to avoid confusion the error was not corrected, but there appears to be no valid reason for perpetuating this mistake. It is, however, recognized that the taxonomic position of this genus is not definitely settled. There are certain resemblances to the genus *Brucella*, such as the presence of cocco-bacillary forms and the limited biochemical activity, but, in view of the irregular morphological picture, it is generally considered that this genus represents a stage between the *Mycobacteria* and the *Actinomyces*.

The first and only accepted member of this genus to be described was *Pf. mallei*, or the glanders bacillus, which was isolated by Loeffler and Schütz in 1882 from a horse dying of acute glanders. A similar organism, *Pf. whitmori*, was later isolated by Whitmore (1913) in India from a glanders-like disease of man, which was termed "melioidosis" by Stanton and Fletcher (1925). It has been tentatively included in this genus by many workers, but it differs from the generic requirements in that it is motile and ferments carbohydrates.

Pf. mallei or the Glanders Bacillus

Morphology. *Pf. mallei* is usually a slender, straight or slightly curved rod with rounded ends, about 0·5 × 2–5μ. It may be arranged singly or in irregular clusters. In artificial cultivation it tends to be shorter and less uniform; in old cultures pleomorphism is marked and long filaments with true branching may be seen. It is non-motile, non-sporing and does not possess a demonstrable capsule. *Pf. mallei* does not stain intensely, and in old cultures may present a beaded appearance; it is Gram-negative and non-acid-fast.

Cultural Characteristics. The glanders bacillus grows readily but slowly on the ordinary media. The optimum temperature is 37° C., range 20°–42° C.; the reaction of the medium is perhaps optimal when slightly acid, but multiplication occurs over a wide range of reaction around pH 7·0. It is a strict aerobe.

In *fluid media*, such as broth, after incubation at 37° C. for 2 days there is a moderate turbidity with a light deposit. No surface growth occurs until 7–14 days, when it is of a slimy, ring-like nature; the deposit also becomes viscid and difficult to disintegrate.

On *solid media*, such as agar, after 2 days' growth at 37° C. the colonies are small, translucent, slimy and greyish-yellow, becoming, with age, opaque, granular and yellowish-brown.

When grown on potato a characteristic pigment is produced, at first it is slightly yellowish or brownish and is said to resemble clear honey, but later it becomes reddish-brown and perhaps chocolate.

Resistance. *Pf. mallei* is easily killed by destructive agents; it is destroyed by heat at 56° C. in 15 minutes. Stock-cultures tend to die rather quickly even when left at room-temperature, and subculture should be done every 2–4 weeks.

Biochemical Activity. Little biochemical activity is displayed. It is said not to ferment carbohydrates, but several observers have noted that glucose is fermented by some strains with the production of acid, but not gas; this action is, however, inconstant. Proteolytic activity is also very poor; indole is not formed and gelatin is not liquefied. Litmus-milk is rendered slightly acid. No hæmolysin is produced.

Serology. The serological reactions of *Pf. mallei* have not been extensively studied. By means of agglutination, agglutinin-absorption and complement fixation it was found that the antigenic structure of the various strains examined was not homogeneous. There were at least two main sub-groups, one of which had a close relationship with *Pf. whitmori*.

Pathogenicity. *Pf. mallei* is primarily a pathogen of equine animals, in which it gives rise to two clinical conditions—glanders, with involvement of the lungs, and farcy, where lesions are present in the skin. Man may become infected with *Pf. mallei*, but such an occurrence is a rarity ; the individuals attacked are mainly those coming in contact with horses or mules.

Experimental infection may be produced in a number of laboratory animals, guinea-pigs being the most susceptible. In the guinea-pig subcutaneous injection of the organism is followed first by abscess formation locally and in the regional glands, then by inflammatory changes in the testicles, in the male animal, with a generalized infection, and, finally, by death usually in 3–4 weeks. Following intraperitoneal injection, progressive swelling of the testicles, commencing in the tunica vaginalis in 2–3 days and becoming purulent, is followed by death usually about the tenth day—this sequence of events is characteristic, and is known as the " Straus " reaction.

Glanders in Man. The human disease is acquired almost invariably as a result of direct contact with an infected animal ; infection from man to man is exceedingly rare. The disease is therefore found chiefly in the attendants of the equine species, and it naturally follows that the incidence of the human condition is greatest where the equine disease is commonest. In Great Britain the introduction of the Glanders and Farcy Order of 1920 has eradicated the disease ; no notification has been made since 1926. The disease, however, is prevalent in other parts of the world, particularly in Russia and Asia. Several cases have been reported in laboratory workers, who have been working with the organism, and consequently the organism should be treated with the greatest respect.

Infection occurs either by the cutaneous route or by the nasal mucosa, and the course of the disease may be either acute or chronic. In the acute form there is usually a local nodular swelling with a spreading pustular skin eruption and foci in the lungs and other organs. This is accompanied by high fever, muco-purulent nasal discharge and extreme prostration ; death occurs in 7–10 days. In the chronic form subcutaneous and intramuscular nodules, becoming purulent, with enlargement of the regional glands and foci in the respiratory tract and other organs, are usually present. The course is long and complete recovery is rare ; cases of apparent recovery, in which there have been latent periods of 1–5 years, have been reported, but in practically all cases a recurrence eventually takes place.

Diagnosis. The chief bacteriological methods employed in the diagnosis of glanders are (1) microscopy—rarely satisfactory alone, (2) the isolation of the organism and its reaction on potato, (3) pathogenicity, with the demonstration of the "Straus" reaction, and (4) application of allergic tests. In the case of closed lesions, where contamination is not present, the first three methods can be readily applied, but if the material is grossly contaminated some difficulty may be experienced.

The allergic test is similar to the tuberculin test in tuberculosis, and it has been widely employed in veterinary practice. The substance, *mallein*, is prepared in the same way as tuberculin, and is usually injected subcutaneously; if glanders is present, definite signs of reaction (local, focal and general) are observed in 10–24 hours.

Serological tests, agglutination and complement-fixation, have also been employed, and it has been found that the most reliable results are obtained by the complement-fixation test.

Prophylaxis and Therapy. The principal prophylactic measures have been directed to the elimination of the disease in horses. The various orders introduced in this country have been particularly satisfactory. Attempts have been made to produce an active immunity to glanders, but without much success.

Mallein has been employed in the therapy of both the human and animal diseases, but in view of the spontaneous retrogressions that are found in the chronic form the results have been difficult to evaluate.

Pf. whitmori : Melioidosis

Pf. whitmori is the ætiological agent of a rare tropical disease, melioidosis, which has been recognized only in Burma, Indo-China, Ceylon and British Malaya. The disease runs an acute course and appears to be of a septicæmic or pyæmic nature; purulent foci are present in many organs. It is usually fatal in 3–4 weeks. Epidemics have been reported in rodents, particularly guinea-pigs, rats and rabbits. No evidence has been obtained of direct infection; it appears that the disease is primarily one of rodents, from which infection passes to man who is relatively resistant.

Pf. whitmori behaves in many respects as *Pf. mallei*. It is Gram-negative and bears some morphological resemblance to *Past. pestis*, but it is motile in young cultures. It grows readily on the ordinary laboratory media; it gives a creamy growth which later deepens to chocolate, on potato, and does not form indole, but liquefies gelatin. Acid, but not gas, is formed from several carbohydrates, including lactose, glucose and mannite. *Pf. whitmori* forms a homogeneous serological group, which appears to be closely related to one of the *Pf. mallei* groups.

CHAPTER XXVII

ACTINOMYCES: ACTINOMYCOSIS: MADURA DISEASE

Actinomyces is the generic term now generally given to a group of organisms which have also been designated "Streptothrix" and "Nocardia". These organisms represent the higher bacteria and stand in an intermediate position between the bacteria and the moulds. The genus was defined by the American Committee (1920) in the following manner: organisms growing in the form of a much-branched mycelium, which may break up into segments that function as conidia. Sometimes parasitic, with clubbed ends of radiating threads conspicuous in lesions in animals. Some species are micro-aerophilic or anaerobic; non-motile.

The term *actinomyces* was first applied to this group by Harz in 1877. Harz found in the pus collected from cattle suffering from "lumpy jaw", a condition now known as actinomycosis, some granules which consisted of slender, branching filaments the ends of which were frequently swollen. The organism has been designated *Actinomyces bovis*, although, in view of the tendency to form radiating filaments in the tissues, it is sometimes referred to as the "ray fungus". Since then a number of species have been described, some being pathogenic to man and animals, while others are saprophytes frequently present on grass, in soil and in the air.

These organisms have been sub-divided into two main classes: (1) the anaerobic species, which include *Actinomyces bovis*, and (2) the aerobic species, which are further split up into (*a*) the acid-fast and (*b*) the non-acid-fast types, which include *Actinomyces maduræ*, the ætiological agent of the tropical disease, Madura foot. The aerobic group contains a number of the common saprophytic members.

Only two species, *A. bovis* and *A. maduræ*, are important human pathogens, but certain other members of the genus may occasionally be present as secondary invaders in lesions of the skin and upper respiratory tract. In the case of the acid-fast types the differentiation of these from tubercle bacilli, particularly in respiratory lesions, may be necessary owing to their possible morphological similarity. The failure of many earlier workers to distinguish the saprophytic members from *A. bovis* has been responsible for much confusion in the study of these organisms, and for this reason many of the early observations on the ætiology of actinomycosis are now valueless; in fact, our knowledge of this genus is still far from satisfactory.

Erikson (1940) carried out extensive investigations on some 20 strains of pathogenic anaerobic actinomyces from human and bovine sources and concluded that human strains could be clearly differentiated from those of bovine origin on morphological, cultural and serological reactions. She suggested that the term *A. bovis* should be reserved for the bovine strains and that human strains should be called *A. isrœli.* In view of the incompleteness of some of the investigations, it is hoped that many more strains will be subjected to similar detailed examination.

A. bovis

Although *A. bovis* was described in 1877, it was only isolated in 1891 by Wolff and Israel.

Morphology. The morphological picture presented by *A. bovis* in the animal tissues is frequently quite distinct from that seen in culture, but in both cases pleomorphism is marked. In culture rods of varying size are usually seen together with branching filaments, clubs and coccal forms; the staining tends to be granular and irregular, and bears some resemblance to that exhibited by the *Corynebacteria.* In the animal tissues the organisms grow as irregular masses in the form of characteristic, small, yellowish granules—the so-called "sulphur granules"; these on microscopical examination are seen to consist of interlacing, filamentous, Gram-positive mycelia, the peripheral ends of which are disposed radially and are swollen, club-shaped and Gram-negative. The nature of these peripheral radial "clubs" has not been determined; they appear to consist of material deposited around a filament and probably represent a reaction of the organism to the tissues. They are not generally found in culture.

A. bovis is non-motile, non-sporing and non-acid-fast; it is Gram-positive. In the tissues the clubbed ends are, however, Gram-negative. Clubs are more prominent in lesions of cattle than in those of man.

Cultural Characteristics. *A. bovis* is able to grow on ordinary media under anaerobic or micro-aerophilic conditions, but growth is poor and slow ; it can, however, be improved by enrichment of the media with blood, serum or glucose.

In a glucose-agar shake-culture maximum growth is found in the second centimetre below the surface ; this indicates that the organism is a micro-aerophile rather than a true anaerobe. The optimum temperature is 37° C. and the optimum reaction of the medium *p*H 7·2–7·6.

In *fluid media*, such as broth, after several days' incubation at 37° C. there is poor growth in the form of a white, nodular deposit resembling puff-balls ; there is neither turbidity nor surface growth.

On *solid media*, such as agar, incubated anaerobically at 37° C. for 7 days a poor growth is seen in the form of small, greyish-white, opaque, convex colonies.

Resistance. *A. bovis* behaves as other non-sporing organisms to the action of destructive agents. It is killed by heat at 60° C. in 10–15 minutes. Stock-cultures should be kept at 37° C. in glucose-agar shake-cultures ; they require frequent subculturing, generally about every 1–3 weeks.

Biochemical Activity. The biochemical activity of *A. bovis* is of little practical value. Several " sugars ", including glucose and lactose, are fermented with the production of acid, but not gas. Gelatin is not liquefied and indole is not formed. Litmus-milk is unchanged.

Serology. Little attention has been directed to the serology of this group and our knowledge is very limited. Erikson has claimed that human strains of *A. bovis* can be differentiated from strains of bovine origin by serological tests.

Pathogenicity. *A. bovis* is pathogenic for cattle and man, producing a disease known as actinomycosis. Laboratory animals are resistant to infection, and localized, regressive lesions only are produced on injecting the organism subcutaneously.

Actinomycosis

Actinomycosis is a relatively common disease of cattle, and although rare in man it is widely distributed, particularly in individuals in contact with cattle. The first detailed account of the disease was given by Bollinger (1877), who examined cases of the so-called " wooden tongue " in cattle and found the lesions were of a granulomatous nature and contained a number of yellowish granules. This condition he termed "actinomycosis". In cattle the lesions are frequently confined to the jaw and soft parts of the head and neck.

In man actinomycotic lesions are usually situated in one of three regions—head and neck, the thorax or the abdomen. The mouth is a common primary site. The mortality rate varies with the site of infection; facial lesions are usually less fatal than the other types. A chronic abscess with marked proliferation of the connective tissue forms and spreads locally by direct extension; the infection later may generalize by way of the lymphatic or blood-streams; the lymph glands are, however, seldom attacked. Thick viscid pus containing the yellowish "sulphur" granules is usually discharged from the broken-down central portion.

The epidemiology and pathogenesis of the disease are unknown. The source of infection has not been determined, but it is ultimately the saliva and nasal discharges of cases, probably animal. It is, however, frequently impossible to trace a human infection to an animal source, and there is no evidence that the disease is contagious in man. Carriers may possibly be responsible for the transmission of the infection in many cases; supporting this theory is the fact that organisms closely resembling *A. bovis* have been isolated from the alimentary tract of healthy individuals. It was at one time considered that infection was transmitted by the agency of grasses and cereals, but the evidence supporting this theory is unconvincing. It has never been proved that *A. bovis* is able to survive outside the animal tissues for any appreciable time; moreover, the organism, which some workers isolated from grass and cereals and considered to be responsible for the condition, has been found to be a harmless saprophyte and not *A. bovis*. While the method of transmission is uncertain, it appears probable that the infectivity for man of *A. bovis* is slight and that some predisposing factor, such as trauma, is necessary to enable infection to take place.

Diagnosis. Examination should be made for the presence of the "sulphur" granules in the thick pus; on rolling the tube containing the pus the granules usually adhere to the sides and can be readily detected. The granules are removed and examined, stained and unstained, after crushing; they are generally soft and can be crushed without difficulty. The interlacing Gram-positive mycelia with Gram-negative clubs are seen.

Cultures should be prepared from the granules after they have been washed in sterile saline. A useful method is the glucose-agar shake-culture, in which the zone of maximal growth appears about 1 cm. below the surface of the medium. The colonies should be removed and examined microscopically. It is interesting to note that there is usually found in association with *A. bovis* a small, Gram-negative, non-motile cocco-bacillus which has been termed *B. actinomycetum comitans*.

Material sent for examination is generally grossly contaminated and may contain saprophytic actinomyces ; care must be taken to differentiate these from *A. bovis*, which is essentially microaerophilic.

Madura Disease

Madura disease is a chronic granulomatous disease bearing a close resemblance to actinomycosis and chiefly affecting the foot ; it is frequently referred to as Madura foot. It is caused by *A. maduræ* and is found mainly in India. Irregular nodular swellings are found in the infected region ; these break down and form abscesses. Later thick yellowish pus is discharged and this contains greyish or yellowish granules, which are larger than those found in actinomycotic pus. The granules consist of interlacing mycelia, which are Gram-positive and non-acid-fast. Growth in culture is pinkish and occurs readily under aerobic conditions ; these points differentiate *A. maduræ* from *A. bovis*. The disease has not been successfully reproduced in experimental animals.

There is another variety of the disease in which the granules are black and consist of interlacing segmented mycelia ; cultures, however, give a growth of a mould, which is quite distinct from *A. maduræ*.

Actinobacillosis

A condition in cattle has been reported which occurs as an epizootic and presents a distribution of lesions which is in the main similar to that found in actinomycosis ; this is termed "actinobacillosis". The ætiological agent is a Gram-negative, non-motile, cocco-bacillus, to which the name *Actinobacillus lignieresi* has been given.

This condition is exceedingly rare in man, but one case appears to have been reported in South America.

CHAPTER XXVIII

BACILLUS: ANTHRAX

THE term *bacillus* has given rise to much confusion, as it has been employed for two distinct purposes: viz., (1) as a general term to indicate all rod-shaped organisms and (2) as the generic designation for a particular group of rod-shaped organisms. Although this dual application appears to be far from satisfactory, it is recommended that the term should be retained as a generic title. When so employed it is a proper noun and must commence with a capital letter; used in the loose sense it is a common noun and is not capitalized. In this way confusion is avoided.

The American Committee have given the following features as indicating this group: aerobic forms; mostly saprophytes; liquefy gelatin; often occur in long threads and form rhizoid colonies; form of rod not greatly changed at sporulation.

One member, *B. anthracis* or the anthrax bacillus, is an important pathogen of animals and man. It was first described in detail in 1877 by Koch, who definitely established its relationship to anthrax. This organism had, however, been observed by several earlier workers, including Davaine, who suspected this pathogenic *rôle*. Many other species have also been isolated, but these have little pathogenic activity and are usually present as harmless saprophytes. A number of these have interfered with the isolation and identification of *B. anthracis* and have been referred to as "pseudo-anthrax bacilli". The original classification of the "pseudo-anthrax bacilli" does not appear to be of any particular value, and many workers consider that all organisms which might be confused with the anthrax bacillus should be included. The most important members of this group are *B. subtilis*, *B. mesentericus*, *B. mycoides* and *B. megatherium*. These organisms usually lead a saprophytic existence, being present in dust, soil, water, hay, etc.; they are consequently frequently found in the body-tissues as contaminants, secondary invaders and, in a few instances, as primary agents in mild localized lesions such as conjunctivitis.

B. anthracis or the Anthrax Bacillus

Morphology. *B. anthracis* is usually seen as straight or slightly curved rods, 0·4 × 8μ, with truncated ends; single forms are common but, under artificial cultivation, chains or unjointed filaments may be found. Spores are formed in culture, but not in the animal tissues, and are central or subterminal and do not bulge. A well-marked capsule is formed during growth in the animal body, but it is lost on cultivation on artificial media unless these contain much animal protein. It is non-motile. *B. anthracis* is Gram-positive and non-acid-fast. The capsules, when present, may be demonstrated in films without difficulty by McFadyean's method, in which an old 1 per cent. aqueous solution of a polychromatic methylene-blue is used.

Cultural Characteristics. Growth takes place readily on the ordinary media and is not greatly improved by the addition of blood, serum or glucose. The optimum temperature is about 37° C., range 14–43° C., and the optimum reaction is *p*H 7·5–7·8. It is a facultative anaerobe.

In *fluid media*, such as broth, after an initial slight turbidity a flocculent deposit forms and the supernatant fluid becomes clear; there is no pellicle-formation.

On *solid media*, such as agar, the colonies present a characteristic irregular appearance, being opaque, greyish-white, with a reticular structure. The surface is uneven and has a "roughish" appearance; on examination with a low magnification lens interlacing chains of bacilli are found. The colonial appearance has been likened to the coils of a barrister's wig. When the growth is confluent a greyish-white pellicle is formed with irregular edges and an uneven surface. On prolonged cultivation on artificial media, smooth, glistening, regular colonies may be formed; these "smooth" types are relatively avirulent.

On blood-agar some slight hæmolysis is frequently present. Spores are not formed in the body-tissues, as they require the presence of free oxygen. They also are not produced when growth takes place at the extremes of temperature, *i.e.*, above 42° C. or below 16° C.; when substances such as phenol (1/800) or potassium bichromate (1/2000) are added to the media a permanent asporogenous strain may be produced.

Resistance. The resistance offered to bactericidal agents by the vegetative forms is similar to that exhibited by the non-sporing organisms; they are killed by heat at 56° C. in 1 hour.

The spores offer a much higher degree of resistance, but are usually killed by boiling at 100° C. for 10 minutes or by a 4 per cent. solution of potassium permanganate in 15 minutes. Of the chemicals employed as bactericidal agents the oxidizing substances have proved to be most efficacious.

Stock-cultures are conserved without difficulty at room-temperature; spores in the dry state remain alive for years.

Biochemical Activity. The fermentation reactions of *B. anthracis* are not of much practical value; glucose, maltose, sucrose and salicin are fermented with the production of acid, but not gas. Indole is not produced and gelatin is slowly liquefied. The type of growth in a gelatin stab-culture is a useful differential point; filaments are formed at right angles to the line of the stab, particularly at the top of the culture, and an inverted fir-tree appearance is thus produced. This is lost as liquefaction progresses downwards from the surface. Litmus-milk is first coagulated and then decolorized and digested.

Serology. Serological tests have not yet been widely employed in the study of this genus. Precipitins, opsonins, agglutinins and bactericidins have been demonstrated, but only precipitation is used to any extent for identification. There appears to be no correlation between the presence of antibodies in an animal's serum and its resistance to infection. The mechanism of immunity in anthrax is obscure.

Pathogenicity. The anthrax bacillus has a high degree of pathogenicity for all classes of animals, but the carnivorous species are usually the most resistant. Birds are relatively insusceptible. It gives rise to spontaneous disease mainly in cattle and sheep, from which man becomes infected.

Laboratory animals, such as guinea-pigs, mice and rabbits, are readily infected by subcutaneous or intra-cutaneous injection of the bacilli. Following the subcutaneous inoculation of a small dose of bacilli guinea-pigs die in 24–48 hours; on autopsy there is usually a localized hæmorrhagic œdema with enlarged, congested regional glands and enlargement and congestion of the spleen, which is dark and friable. The blood is dark and usually contains large numbers of bacilli.

It has been found that there is a definite relationship between capsule-formation and virulence, which is quite independent of spore-formation. Capsulated strains, which give the typical irregular or roughish colonies, are the most virulent. Avirulent strains tend to form small smoother colonies, in which chains of bacilli or filamentous forms are not found. The production of a toxin is uncertain; if present it behaves as the endotoxins rather than the exotoxins.

Anthrax

Anthrax occurs primarily in animals and secondarily in man as an occupational disease. It has long been recognized and has played an important part in the development of bacteriology and immunology. Pasteur's early attempts at immunization in the field were aimed at protecting sheep against this disease.

Anthrax in animals is a widespread disease, being particularly prevalent in Russia and parts of Asia; it is encountered in the herbivora, particularly in cattle, sheep, goats, horses and pigs; Algerian sheep are, however, relatively resistant. In view of the frequent enlargement of the spleen, the disease has been called "splenic" fever. The infection may be spread directly from animal to animal or may enter the body *viâ* the alimentary tract, being ingested with the food. A septicæmia develops and the bacilli are excreted in the saliva, urine and fæces; death supervenes in the majority of cases a few hours to several days after the onset of symptoms. Flies have occasionally transmitted the disease following contamination with the blood or excreta of an infected animal. It is interesting to note that in this country single cases are usually reported; infection is considered to occur by way of the intestinal tract, the organisms, as spores, being present in imported foods. The course is generally short, death occurring in 12–48 hours after the onset.

In man there are three portals of entry—the skin, the respiratory tract, and the alimentary tract; the latter form is, however, a rarity.

The *cutaneous* form occurs mainly in people handling infected meat or some product of an infected animal such as hair, hide, wool, etc. It is the commonest form of the disease and is usually encountered in butchers, farmers, slaughterers, tanners, brush-makers, etc. It may also arise from the use of a shaving brush with contaminated bristles. The spores are implanted directly on to the skin, through which they pass by way of cuts, minute abrasions or the hair follicles. A local lesion is produced which has been termed a "malignant pustule". This term is actually a misnomer, as pus-formation is not usually a prominent feature and also the condition is not particularly malignant. The incubation period is short, about 24 hours, and is followed by the appearance of a local papule, which increases in size, and by the third or fourth day becomes a red indurated mass with a central, necrotic black area which is surrounded by an elevated zone of small vesicles. Localized œdema surrounding the pustule is frequently present. The mortality rate is low and depends to some extent on the site of the lesion. It is highest where the tissues are loose, as in the neck ; in fatal cases the œdema spreads and septicæmia develops.

The lesions are found almost entirely on the exposed regions. Legge (1934) collected 1,000 cases of anthrax in man of which 937 (141 fatal) were of the cutaneous type ; the main distribution of the lesions and mortality rates are given in Table XXV.

TABLE XXV

Incidence and Mortality Rate of Cutaneous Anthrax
(after Legge, 1934)

Situation	Cases	Mortality per cent.
Forehead	66	3·3
Extremities	209	6·7
Cheek	156	10·9
Eyelid and Eyebrow .	61	19·7
Chin	39	23·1
Lower jaw . . .	30	23·3
Neck	292	24·3

The *pulmonary* form is essentially an industrial disease and is often referred to as " wool-sorter's disease ". It is seen in individuals concerned with the treatment of animal products and in this country cases are found mainly in the Bradford district, where the worsted industry is carried on. When the hair or wool is contaminated with anthrax spores, these are inhaled with the dust produced during the sorting and combing of these products. A generalized infection with septicæmia develops. The mortality rate is extremely high ; Legge reported 54 cases, of which 53 ended fatally. On autopsy the principal changes are discoloration of the skin, widespread cellular œdema and congestion, particularly involving the trachea, the bronchi, where the primary lesion is usually situated, the lungs and the mediastinum. At the primary focus of infection the mucosa is necrotic and ulcers are formed. Multiple subcutaneous hæmorrhages and serous effusions into the pleura, peritoneum and pericardium are also common features.

Diagnosis. Bacteriological methods are required not only to diagnose anthrax in man and animals, but also to detect the presence of the bacilli or spores in suspected material, such as shaving brushes, wool, hair, etc. When anthrax is suspected in animals an autopsy is illegal in this country, as by such a practice the bacilli are liberated and become a source of infection for other animals. A swab soaked in the blood of the animal and collected with due precaution or an ear of the animal should be submitted for examination.

In the human cutaneous form, the bacilli are present in greatest numbers in the vesicles surrounding the black central eschar. The vesicular fluid should be collected either in a capillary pipette or on a swab. In the pulmonary type the sputum and blood should be examined, and in the intestinal form the vomit.

The methods employed in identifying the organism are microscopy, culture, pathogenicity and sometimes serology. In *microscopy* the presence of capsules and absence of motility are points of most significance. *Cultural tests* are carried out on agar, blood-agar plates and in a gelatin-stab to obtain the inverted fir tree appearance. For the *pathogenicity* tests guinea-pigs or mice are usually employed. With pure cultures subcutaneous injection is used, but with grossly contaminated material the most valuable procedure is cutaneous scarification. As the penetration of a few anthrax bacilli is sufficient to produce infection, the organism can frequently be isolated by this technique. The most useful *serological test* is the thermo-precipitin test of Ascoli. In this test extracts of material or infected tissue are tested against an immune serum. Although the reaction is not absolutely specific, the specificity becomes serviceable by suitable dilution of the reagents.

In the demonstration of anthrax bacilli on shaving brushes, wool or hair, the suspected material is soaked in a weak solution of KOH (3–5 per cent.), incubated for a few hours and then heated at 80° C. for several minutes; after this treatment cultures are prepared and the material should also be injected subcutaneously or rubbed on the scarified skin of a mouse or guinea-pig.

As saprophytic members of this genus are frequently present along with the anthrax bacilli, the identification of the latter requires great care. Useful differential features are given in Table XXVI.

TABLE XXVI

Differentiation of B. anthracis from the Pseudo-anthrax Bacilli

Test	*B. anthracis*	Pseudo-anthrax bacilli
(1) Capsules.	Yes (in the tissues).	No.
(2) Motility.	No.	Yes.
(3) Gelatin.	Inverted fir tree growth; liquefaction slow.	No fir tree growth; liquefaction rapid.
(4) Pathogenicity for laboratory animals.	Marked.	Absent or very slight.
(5) Precipitin.	Strongly positive.	Weakly positive.

Prophylaxis. Human anthrax is dependent on the presence of the animal disease, and is controlled mainly by attention to industrial hygiene. The incidence of the condition has decreased to a marked degree in this country following the introduction of regulations which aim to prevent the infection of animals and to ensure the disinfection of material likely to be contaminated. Many regulations, such as the Anthrax Order of 1928, have been introduced in this country to reduce the possibility of animals becoming infected. The disease is notifiable, animals with anthrax are immediately destroyed and post-mortem examinations are illegal. The carcases are either buried in a deep pit surrounded by lime or burned. If through a mistaken diagnosis an autopsy is carried out, the carcase should be burnt without delay and the buildings thoroughly treated with disinfectants.

Suspected material, such as wool or hair, introduced into this country, is subjected to treatment at Liverpool by the "Duckering" process. After preliminary treatment with alkalies, the material is acted on by a 2 per cent. solution of formaldehyde at 102°–105° F. for 20 minutes; it is then washed in water and dried by air at 160° F. The treatment of shaving brushes and hides is more difficult and no generally accepted method is available.

Further preventive measures include the early notification and prompt medical attention of all suspicious lesions and the education of the workers to the potential dangers of the work.

Specific measures have not been applied to man, but various vaccines have been employed extensively in combating the disease in sheep and cattle on the Continent. Organisms attenuated by cultivation at 42° C. are used for the first injection, and less attenuated ones for the second dose, as originally recommended by Pasteur. The results are not always satisfactory, as it is impossible to standardize the virulence of the organisms. Another disadvantage is that the induced immunity is of relatively short duration.

Therapy. Treatment in man usually takes the form of the internal administration of either arsenical compounds (*e.g.*, salvarsan), which are used extensively in South Africa, or Sclavo's serum. The results have been most favourable in the cutaneous form. Sclavo's serum is prepared in the ass and has been used mainly on the Continent, particularly in Italy. Large intravenous doses (50–100 c.c.) should be given daily until the general condition improves. While this serum has given satisfactory results, its mode of action is unknown ; an opsonic action has been suggested, but this is unlikely to be the complete explanation.

Preliminary observations indicate that the sulphonamides may be valuable therapeutic agents in anthrax, but conclusive evidence has not yet been obtained. *B. anthracis* is sensitive to penicillin, but results of clinical trials are as yet few and inconclusive.

In the cutaneous form surgical intervention is usually unnecessary, and is perhaps dangerous, as it may facilitate the introduction of the bacilli into the blood-stream.

CHAPTER XXIX

CLOSTRIDIUM: TETANUS: GAS GANGRENE: BOTULISM

The term *Clostridium* has been employed by different workers as the generic name for various species of the spore-bearing family "Bacillaceæ". The American Committee (1917) used it to designate all the spore-bearing anaerobes irrespective of the position of the spore, and it is now generally used in this sense. The following description was given: anaerobes; often parasitic; rods frequently enlarged at sporulation, producing clostridium or plectridium forms.

Members of this group were recognized in the early days of bacteriology. Pasteur and Koch both described pathogenic and non-pathogenic species, but the methods of examination were not very satisfactory; fluid cultures only were used and the inherent difficulties involved in the study of this group were not fully appreciated. It is, therefore, not surprising that results of this early work soon reached a state of chaos. Species were isolated, subjected to an incomplete examination and, in many instances, unjustifiable claims of the discovery of fresh species were made. During the First Great War, when cases of gas gangrene were seen with great regularity, an added impetus was given to the study of this group. Greatly improved technical methods were introduced and the use of solid media became practicable. Several valuable reports resulted, and the knowledge of this genus was gradually freed from confusion. It was found that different strains of one species had frequently been given quite distinct names and were considered as separate species; *e.g.*, *Cl. welchii* had been styled by different bacteriologists—*B. welchii*, *Bacillus of Achalme*, *B. aerogenes capsulatus*, *B. phlegmonis emphysematosæ*, *B. perfringens* and *B. enteritidis sporogenes*.

The early discrepancies are easily explained by the difficulties involved in obtaining pure cultures, particularly with the methods then in use. These organisms appear to grow as well, if not better, in mixed as in pure cultures; the spores also tend to adhere, and when different varieties are present the individual forms may be exceedingly difficult to separate. Irregularities were obtained mainly in the study of gas gangrene, in which condition gross contamination of the wound is invariably found. Pathogenic and non-pathogenic species are generally present and the identification of the individual members is frequently a matter of great difficulty.

The pathogenic members of this genus have been described in some detail, but the study of the saprophytic species is far from complete. Some have been accurately defined, but it appears highly probable that many others have not yet been recognized as separate species.

The natural habitat of most, if not all, members of this genus is considered by the majority of workers to be the soil. They are widely distributed in nature and some are isolated with great frequency from the fæces of man and animals. Their presence in the fæces is thought to result from the ingestion of contaminated foods.

The species important to a medical bacteriologist are those either concerned with the causation of or present in the lesions of gas gangrene, tetanus and botulism. In the former group are *Cl. welchii*, *Cl. septicum*, *Cl. œdematiens*, *Cl. histolyticum*, *Cl. fallax* and *Cl. sporogenes*. Some of the more pathogenic members of this group, and *Cl. chauvœi*, are also responsible for important diseases in animals, *e.g.*, braxy, blackleg and lamb dysentery. In the tetanus group are *Cl. tetani*, *Cl. tetanomorphum* and *Cl. tertium*, and in botulism the various types of *Cl. botulinum*.

These have been tentatively classified according to their morphological and biochemical behaviour (see p. 349).

Morphology. The *Clostridia* are usually large, straight or slightly curved rods, 0·5 × 8μ, with rounded or truncated ends. Pleomorphism is a common feature and a great variety of forms may be seen—filaments, clubs, navicular and citron shapes. In old cultures irregularly staining involution forms are frequent. Motility is shown by most species, but it is frequently difficult to demonstrate; an important non-motile member is *Cl. welchii*. Capsule-formation is only exhibited by *Cl. welchii* and *Cl. butyricum*.

All members form spores which may be demonstrated, in the case of the pathogens, in the body-tissues. Some members, such as *Cl. sporogenes*, spore readily, but others are less consistent, *e.g.*, *Cl. welchii* forms spores only in media free from a fermentable carbohydrate. Variation is encountered in the position and shape of the spore, which, when mature, is almost invariably wider than the vegetative bacillus—hence the term "clostridium". The spores may be terminal, as with *Cl. tetani*, or more commonly equatorial or subterminal.

The anaerobes stain readily with the ordinary dyes. They are Gram-positive, but in old cultures they tend to be decolorized more readily than most Gram-positive organisms. The spores are not stained by these methods and the special spore stains are required for their demonstration.

Methods of Cultivation. The anaerobes fail to grow in the presence of free oxygen, and consequently the usual methods of cultivation are of limited value. Atmospheric oxygen has not, however, any marked destructive action on the organisms. Some of the *Clostridia* are actually micro-aerophiles. The optimum oxygen requirement is thus subject to much variation, but all grow well in the absence of atmospheric oxygen. Under anaerobic conditions oxygen required for growth is obtained from the constituents of the medium. The cultural methods in general use are consequently based on four principles: (1) the prevention of oxygen from reaching the medium or certain parts of it; (2) the removal of oxygen from the air; (3) its replacement by other gases; (4) the addition of active reducing substances to the medium.

(1) The prevention of oxygen from reaching the medium is probably the simplest method of cultivating the anaerobes. The ordinary media are prepared for use either as stab- or shake-cultures in the case of solid media or in deep tubes with fluid media. Surface growth of the anaerobes is not obtained aerobically. Growth is facilitated by the addition to the medium of some reducing substance such as glucose, cysteine hydrochloride or particles of tissue, and by layering the exposed surface with sterile vaseline or paraffin. Before use the media should be heated in boiling water for a short time in order to expel any free oxygen dissolved in the medium. Satisfactory media are glucose-agar or -broth and Robertson's meat medium (*q.v.*).

(2) The absorption of oxygen from the air is readily carried out by placing a small amount of pyrogallic acid on the plug of the inoculated tube or flask, adding some alkali such as KOH, and sealing with paraffin or a rubber stopper. Alkaline solutions of pyrogallol have the property of absorbing large quantities of free oxygen. With this method most media can be used,

as it is possible to obtain surface growth on agar or glucose-a slopes. The air may also be removed by exhaustion with vacuum pump.

(3) For the exhaustion and replacement of oxygen from t air some special apparatus is necessary; that in frequent use the McIntosh and Fildes jar or some modification of it. T jar has a lid through which are one or two apertures, while the under surface is some palladium asbestos. This is contain in a wire gauze, which is connected to two terminals on t outside of the lid. The original jar was glass, but a more servi able form has been made of a copper-alloy; the lid is fixed b number of metal clamps. It is built to hold both plates a tubes (about two dozen tubes in all). After placing the inocula media inside and securing the lid, the air is exhausted by me of a vacuum pump; hydrogen, from either a Kipp apparatus a cylinder, is then slowly introduced, and an electric current passed through the palladium asbestos. This, when hot, a as a catalyst and promotes the union of any residual oxy with hydrogen to form water. When replacement is comple the jar is incubated at the desired temperature. It has great disadvantage in that the contents can only be examined taking down the jar. For further incubation exhaustion replacement must both be carried out again. With the gl jar the tubes are visible and some substance to indicate the deg of anaerobiosis can be added. The usual indicator consists equal parts of N/10 NaOH (6 per cent.), 0·5 per cent. methyle blue (3 per cent.) and 6 per cent. glucose with some thy crystals. This is mixed and boiled immediately before use is colourless in the absence of oxygen.

(4) Growth of the anaerobes can be obtained under ordin atmospheric conditions in fluid media containing active redu substances such as small strips of sterile steel, sodium t glycollate and ascorbic acid. The iron should be added to medium immediately before use as it tends to form a precip Sodium thioglycollate is the active principle of Brewer's me (*q.v.*), which is now widely used in the investigation of anaerobes.

Jars of the McIntosh and Fildes type have yielded the results in the isolation of pure cultures from grossly contami material, for which surface growth on a solid medium, su 6 per cent. agar or blood-agar, is essential.

Cultural Characteristics. The *Clostridia* do not grow ordinary aerobic conditions, but some, such as *Cl. welchii* and *Cl. sporogenes*, multiply under micro-aerophilic conditions. The temperature range is wide, the optimum temperature being usually about 37° C., and the optimum reaction lies between pH 7·0–7·4. As a rule growth, particularly on solid media, is not profuse and media enriched with serum, glucose or blood, or special media such as Robertson's meat medium, are generally used.

On *solid media* growth is poor and slow; the colonies tend to be small, effuse and irregular. With some species, such as *Cl. welchii*, it is stated that characteristic appearances may be found, but considerable experience is required before much reliance can be placed on this criterion. The growth frequently tends to be confluent when the surface of the medium is moist, and some difficulty may be experienced in differentiating it from the medium. This spreading of the colonies can be inhibited by ncreasing the concentration of agar to approximately 6 per cent.

In *fluid media* turbidity with a moderate granular or powdery deposit is produced by most species. In Robertson's meat medium the meat is digested and blackened by the proteolytic members, while the saccharolytic organisms frequently turn it pink and produce bubbles of gas.

On blood-agar plates hæmolysis is exhibited by many members.

Resistance. The spores of the *Clostridia* are relatively resistant o the ordinary bactericidal agents. Variation in the degree of esistance is shown by the individual members. *Cl. botulinum* is one of the most resistant and *Cl. welchii* one of the most susceptible members. All are destroyed in the autoclave at 120° C. n 30 minutes. Stock-cultures may be conserved at room-temperature in the meat medium for some time (6–12 months); n some cases survival for several years has been reported.

Biochemical Activity. The saccharolytic and proteolytic ctivities of the anaerobes are frequently very marked, but it is mportant to note that irregularities are often encountered.

Some members, including *Cl. sporogenes*, *Cl. histolyticum*, *Cl. ifermentans* and *Cl. botulinum*, are highly proteolytic; gelatin, asein, serum and fibrin are all attacked and meat is digested nd blackened. Others, including *Cl. chauvei*, *Cl. septicum*, *l. welchii*, *Cl. tetani* and *Cl. œdematiens*, are less active. They quefy gelatin, but do not digest serum, fibrin or meat. A umber, such as *Cl. fallax* and *Cl. tertium*, exercise very little roteolytic activity and do not even liquefy gelatin.

Saccharolytic activity is characterized by the production o large amounts of gas, which is usually carbon dioxide an hydrogen. *Cl. welchii* is most active in this respect, but C *septicum*, *Cl. chauvœi*, *Cl. œdematiens* and *Cl. fallax* also exhib marked saccharolysis. Glucose, maltose, lactose and sucros are the sugars frequently fermented. *Cl. tetani* has no sacchar lytic activity.

Litmus-milk manifests changes which are useful in distinguish ing the various members. Peptonization and digestion ar produced by the proteolytic members, acid and clot by th saccharolytic organisms, which may also form gas. *Cl. welch* frequently gives the characteristic appearance known as "storm fermentation", in which the clot is broken up by the rapid an abundant gas production.

Serology. The serological reactions of the anaerobes have n been investigated in any great detail. Agglutination, precipit tion, complement-fixation and the toxin-antitoxin reactio have been carried out in many instances, and the best resul have been obtained by the agglutination and toxin-antitox reactions. By agglutination many members have been su divided into a number of sub-groups. With the motile strai both O, or somatic, and H, or flagellar, antigens are involve It appears that the H antigen is responsible for the typ specificity and the O factor for the species-specificity. "Smooth to "rough" (S → R) variation is also responsible for irreg larities. The clostridia are not antigenically homogeneous.

It is of interest to note that in most instances (*e.g.*, *Cl. tetani*) t various serological groups of a given species produce the sa toxin. *Cl. botulinum*, however, is different in this respect; ea of the three recognized types (A, B and C) forms a separa toxin.

Toxin Production. The pathogenic anaerobes owe their path genicity almost entirely to their capacity for producing toxi These toxins possess the properties of the "exotoxins". So are extremely potent: *e.g.*, *Cl. tetani* kills a mouse with a dose 0·00001 c.c.; *Cl. botulinum* a mouse with $1/10^6$ c.c.; *Cl. œdem tiens* a mouse with 0·0002 c.c. Others are less active; the leth dose for the mouse of *Cl. welchii* toxin is about 0·25 c.c. *botulinum* toxin is relatively heat-stable and requires a temperat of 80° C. for ½ hour for its destruction. The special properties a action of the individual toxins are considered later.

There are several types of *Cl. welchii*: type A is concerned w gas-gangrene, the others (types B, C and D) are responsible for diseases in sheep.

Cl. welchii A forms several toxic substances, in particular the alpha (α) toxin and theta (θ) hæmolysin, as well as hyaluronidase; the α toxin, however, appears to be the important pathogenic factor.

Pathogenicity. While members of this genus are important pathogens of man and animals, the anaerobes are not strictly parasitic; they are actually unable to multiply in living tissues and only produce infection when implanted in tissue which has been damaged or destroyed in some way, as by trauma. In man the main diseases produced are tetanus, gas gangrene and botulism; in animals they give rise to braxy, blackleg and lamb dysentery. In all instances the clinical picture is mainly due to the activity of the toxin produced by the causative organisms. Botulism in fact is entirely an intoxication; the toxin is produced outside the body and is absorbed from the intestinal tract after ingestion with food.

Experimental infection can be produced by the more pathogenic members of the gas gangrene group by the injection of whole cultures; washed spores or bacilli have no such effect. The most satisfactory animals are guinea-pigs, mice and pigeons; death follows the intramuscular injection of a culture in 24–72 hours. The post-mortem appearance given by the different species varies, but local swelling, œdema, hæmorrhage and gas

TABLE XXVII

Classification of Certain Members of the Genus Clostridium

BIOCHEMICAL ACTIVITY

Position of spore	Both P. and S.		Slight P. No S.	S. No P
	P. > S.	S. > P.		
Central or subterminal	*Cl. sporogenes* *Cl. histolyticum* *Cl. botulinum*	*Cl. welchii* *Cl. septicum* *Cl. œdematiens* *Cl. chauvœi*		*Cl. fallax*
Terminal			*Cl. tetani*	*Cl. tetanomorphum* *Cl. tertium*

S = Saccharolytic activity.
P = Proteolytic activity.

production with necrosis, redness and œdema of the local muscles are frequently found.

Most laboratory animals are susceptible to both *Cl. tetani* and *botulinum* toxins; mice and guinea-pigs are those frequently employed in experimental and routine work.

Classification. A final sub-division of the anaerobes is not possible as our knowledge is still far from complete. A tentative scheme, based on morphological and biochemical criteria, has been introduced and provides a useful means of sub-grouping the members at present recognized. The arrangement of the more important members is given in Table XXVII.

Tetanus

Tetanus is a disease of antiquity and is now widely distribute throughout the world. Its incidence is, however, relatively low considering the great possibility of infection with *Cl. tetani* which has been isolated with frequency from soil and the faeces of man and animals. The spontaneous disease is encountered in man and various animals, particularly the horse. The relationship of *Cl. tetani* to tetanus was definitely established in 1889 by Kitasato, who isolated the organism in pure culture from the tissues of the wound and reproduced the condition experimentall with the pure cultures. Earlier workers, including Rosenbac (1886), had reproduced the disease experimentally by the injectio of material collected from the local lesion, but although organism resembling *Cl. tetani* were seen microscopically this organism wa not isolated in culture.

In man the majority of cases have followed wounds contaminated with soil and manure, such as those occurring in war o accidental traumatic lesions. Cases have also occurred in newly born infants following infection of the umbilical cord, and afte such operations as criminal abortion, the hæmostatic injectio of gelatin, vaccination, surgical operations and teeth-extraction In cases of post-operative tetanus the catgut is generally hel responsible for the introduction of the tetanus spores; great car is therefore necessary in the preparation of catgut, the sterilisation process should be carried out with great care. Ma chemical bactericidal agents have been used to sterilise catgut but the results are frequently unsatisfactory.

The tetanus bacillus or spore gains entrance at the time of th injury, and the subsequent events depend largely on the loca environmental conditions. If there is little tissue damage an no contamination with foreign material, the spores may either undergo phagocytosis or remain dormant for some time. The wound heals and the host does not develop tetanus until the general or local resistance is lowered by some other infection or local trauma. This type of infection has been termed "idiopathic tetanus". When, however, the implantation of the organism or spore is accompanied by tissue damage and gross contamination of the wound the organism is able to multiply. The bacilli grow locally and toxin is produced.

The pathogenesis has been investigated in some detail in experimental animals, particularly rabbits, guinea-pigs and mice. The results indicate that the toxin, produced locally, passes to the nerve-cells of the anterior horn of the cord by way of the axones or perineural lymphatics of the regional motor nerves. This gives rise to the tonic muscular contractions characteristic of tetanus. The nerve-cells corresponding to the area of infection are usually the first to be involved, after which the toxin passes to other parts of the cord. The toxin enters the nerve by the exposed nerve-endings or by breaches in the sheath caused by the trauma. Some toxin also passes into the blood-stream, but this is not usually the route of transport to the cord. The bacilli remain localized, and their main activity is to produce the toxin.

In laboratory animals two types of tetanus have been described —ascending and descending—but these are frequently combined. The ascending type is demonstrated by injecting mice or guinea-pigs subcutaneously into one of the hind limbs. The toxin passes by the local motor nerves to the central nervous system, through which it subsequently diffuses. The spasms are first observed in the injected limb, then in the corresponding limb and later in the upper limbs and head.

The descending form is seen after intravenous inoculation of the toxin, which gains entrance to the central nervous system probably *viâ* the exposed motor nerve endings. The spasms quickly develop and the upper limbs are usually affected before the lower extremities. It may follow the injection of the toxin by any route in large animals, *e.g.*, the horse.

The production of the infection does not depend solely on the introduction of the tetanus bacilli or spores into the tissues. This must be accompanied by other factors, such as trauma or the presence of gross contamination of the wound. The *rôle* played by these factors has been investigated by various workers, who found that infection did not follow the injection of the washed bacilli or toxin-free spores into mice or guinea-pigs. It occurred, however, on the addition to the washed organisms of such substances as a small amount of toxin, the ionizable salts of calcium, soil or other bacteria. The importance of these additional substances is that they render the tissues of the host favourable for the multiplication of the organisms. Fildes considered that the failure of the washed organisms to grow was due to the presence of oxygen in the normal undamaged tissues. When local necrosis was produced the oxygen tension was reduced and germination of the tetanus bacilli or spores proceeded. The function of the foreign bodies was to produce this local necrosis.

A similar sequence of events is also found in the spontaneous human disease. It thus follows that the incubation period is variable and depends mainly on the nature and site of the local lesion. The average time is about 8 days, but this has been increased by the introduction of antitoxin as a prophylactic measure. The disease is characterized by a series of tonic muscular contractions which are local at first, but later become general. As the muscles of the jaws are frequently involved, the disease has been popularly termed "lock-jaw". The mortality of the disease in the absence of serum-therapy is very high. 80–90 per cent. The most severe cases are those in which the incubation period is short, while tetanus resulting from lesions of the head and upper extremity tends to be more severe than that following injuries of the lower extremities.

Diagnosis. The bacteriological methods used in the diagnosis of tetanus are microscopy, culture and pathogenicity tests.

The important points of the microscopical examination are the presence of motility and the demonstration in the local lesion of rods with terminal spores, giving the so-called "drum-stick" appearance to the organism. This appearance is, however, only suggestive of the presence of *Cl. tetani*, as it is also given by other closely related but non-pathogenic *Clostridia* (*e.g.*, *Cl. tetano-morphum*).

In cultural tests as much tissue as can be obtained from the wound should be submitted for examination. This is added to tubes of Robertson's meat medium, some of which are then heated at 80° C. for 20 minutes to destroy vegetative organisms and all are incubated under anaerobic conditions at 37° C. The tetanus bacillus produces a general turbidity and gas, but does not blacken or digest the meat. Another satisfactory and rapid method of isolating *Cl. tetani* is to inoculate the water of condensation of an agar slope with the tissue heated at 80° C. for 20 minutes. *Cl. tetani* produces a thin effuse growth which spreads upwards on the surface of the medium ; in 24–48 hours the upper edges of the filamentous growth are examined by means of a hand-lens and subcultured on to various media. *Cl. tetani* liquefies gelatin, but neither digests serum nor ferments any sugars.

Pure cultures or suspensions of the original material are finally injected subcutaneously into experimental animals, usually mice or guinea-pigs ; *Cl. tetani* gives rise to characteristic spasms resembling tetanus in man. Conclusive proof of the identity of the organism is obtained by demonstrating the neutralizing action of the specific antitoxin.

Prophylaxis. The value of antitoxin as a prophylactic measure was conclusively demonstrated during the Great War. It was introduced during the last quarter of 1914 as a routine procedure in the treatment of wounded soldiers. The development and progress of the disease were influenced in several ways : (1) The incidence was greatly reduced ; figures collected from the British hospitals in France indicate that in September, 1914, tetanus developed in 9 per 1,000 wounded, but by December, 1914, following the introduction of antitoxin, the incidence was only 1·4 per 1,000. (2) The incubation period was lengthened. The average figure of 10 days in the unprotected was increased to an average of 45 days in the protected individuals. (3) The severity and type were changed ; the disease became more subacute and local tetanus was a more prominent feature.

While there was no doubt about the beneficial effects of antitoxin in prophylaxis, the results were less satisfactory than those obtained in diphtheria. Several factors are responsible for this : (1) the relatively low concentration of the antitoxin in the blood after injection and the possible high toxin production at the local lesion. (2) The shortness of the immunity conferred by the antitoxin and the possibility of tetanus spores lying dormant for some time and subsequently germinating and producing toxin.

The main prophylactic application of antitoxin is seen during war-time conditions, but in civil practice all individuals with wounds, in which contamination with soil or manure has taken place, should receive antitoxin. The dosage employed during the 1914–18 war was four subcutaneous injections of 1,000 international units at intervals of 7 days. In all cases surgical measures

are necessary. The wound must be thoroughly cleaned and
necrotic tissue removed; this renders the focus less favoura
for germination of the tetanus spores. Antitoxin should alw
be given before surgical treatment is commenced.

In the case of individuals liable to receive contaminated woun
e.g. soldiers, active immunization with TETANUS TOXOID is carr
out; this procedure was officially introduced into the British Ar
as a routine measure in 1938. Immunity is produced slowly, l
once developed it appears to be of relatively long duration; t
doses at an interval of 6–8 weeks were originally given. Furt
doses at intervals of 10–12 months are now recommended, as
has been found that the administration of a third dose is follow
by a rapid and marked increase in the antitoxin titre of the ser
and that this increase is maintained for some time. The resu
have been striking; tetanus in the British Army during t
recent Great War was negligible. In the American Army it
interesting to note that toxoid, and not antitoxin, is given
wounded who have been previously immunised, while in t
British Army a single dose (3,000 units) of antitoxin (A.T.S.
still used to reinforce the active immunity.

Therapy. The value of antitoxin as a therapeutic agent
undecided. It is accepted that once the toxin has reached t
central nervous system it is relatively inaccessible. Serum m
therefore be given as early as possible, large doses are necess
and the intrathecal route, supplemented by intravenous or int
muscular injections, should be used. It is therefore import
that cases should be diagnosed in the initial stages and that la
doses of serum should be administered without delay. An in
dose of 32,000–50,000 international units intrathecally w
16,000–20,000 units intramuscularly is suggested by m
workers; this is repeated on the second day, after which
intravenous and intramuscular routes only are used. T
duration of treatment and the dosage depend on the condition
the patient.

The sulphonamides and penicillin have given disappoint
results in the treatment of tetanus.

Preparation of Antitoxin. Antitoxin is prepared by the im
tion of the horse with increasing doses of alum-toxoid. T
selection of a toxin-producing strain of *Cl. tetani* does not pres
any difficulty, although it is important to note that non-to
strains are not uncommon. Eight serological types have b
demonstrated by agglutination, but they all produce the sa

oxin. Broth is seeded and incubated anaerobically at 37° C. or 5–14 days, after which it is passed through a Seitz or Berkefeld lter.

The activity of the toxin is determined by estimating the L.D. on subcutaneous or intramuscular injection of mice or uinea-pigs. For mice of 20 grm. this is, on an average, 0·00005 c., and for guinea-pigs of 350 grm. ten times greater. The tetanus acillus produces two distinct toxic substances—(1) tetano- pasmin and (2) tetanolysin.

The *tetanospasmin* is the more important of the two toxins, nd is the one usually referred to in discussions of the tetanus oxin. It has an affinity for nervous tissue, and is directly sponsible for the symptoms of tetanus. It is extremely active hen injected into the tissues (a quantity as small as 0·000005 c.c. f a broth culture filtrate may kill a mouse), but is inert when given y the mouth ; it is readily destroyed by moderate heat (60° C.), xidizing agents, acids and strong alkalies.

The *tetanolysin* has a lytic action on red blood corpuscles, but robably has little pathogenic significance. It is very labile, eing readily destroyed by heat at 50° C. The so-called "non- oxic" strains of *Cl. tetani* may produce abundant tetanolysin, hile virulent strains may not produce any.

After its activity has been determined the toxin is formolized give the toxoid, which is treated with potash alum, calcium hloride or tapioca. Immunization is slow and may take several onths. The antitoxin is contained in the pseudo-globulin raction of the serum and may be readily concentrated to approxi- ately three times its original value. The standardization in arious countries of the antitoxin content of a serum has lacked niformity, owing to the existence of three different units and ur methods of assay. Attempts are being made to overcome his, and the method now advocated is one in which a satisfactory oxin is standardized against a dried standard antitoxin by etermining a dose analogous to an "L +" dose in a 350 grm. uinea-pig (1/10 of a unit of antitoxin is used and the animal ust die in just 96 hours) ; from the data available the titre of e freshly prepared antitoxin can be determined. This method as originally worked out in America and it was later adopted by e League of Nations Standards Commission ; the adopted ternational unit is, however, one-half the original U.S.A. nit.

Gas Gangrene

Gas gangrene, a common disease in the days of pre-antiseptic surgery, is now a rarity in civil practice and is essentially a war disease. The anaerobes not infrequently give rise to wound infection and may also be responsible for cases of puerperal fever and mild types of enteritis, but the classical cases of gas gangrene are seldom seen.

Our knowledge of this condition is due largely to observations made during the Great War by British and French bacteriologists. Prior to 1914 the bacteriology of the disease was in a state of chaos. Widely divergent reports were made and disagreement was common. This was not surprising, as the technique employed in these investigations was of a primitive nature. The great improvement in technique, particularly the introduction of the McIntosh and Fildes jar, simplified the bacteriological investigations of the disease. The report of the Anaerobic Committee of the Medical Research Council (1919) and the monograph of MM. Weinberg and Séguin (1918) give comprehensive details of some of the various observations carried out on gas gangrene during the war and indicate the factors which caused previous work to be inaccurate and unreliable. It is interesting to note that these findings have been largely confirmed by observations in the recent Great War.

The incidence of gas gangrene is closely related to the liability to contaminated wounds in which extensive tissue destruction and imperfect drainage are present. As already stated, the anaerobes are only able to multiply in devitalized tissue. It thus follows that war-time conditions are more favourable than those prevailing in ordinary civil life. In civil practice the disease is now usually seen as a complication of street or other accidents, in which contamination of the wound with soil or fæces has taken place. In the pre-antiseptic days, where the tissue destruction at the operation was invariably followed by infection of the wound, most surgeons had much unpleasant experience of the condition.

During the 1914–18 war it was noted that cases were most numerous during active operations, when the wounded were not collected for some time and when the possibility of the wounds becoming contaminated with soil was increased. The incidence was also highest following battles fought over highly cultivated and manured ground and in wounds caused by bombs, shrapnel and fragments of high-explosive shells.

The onset of gas gangrene may occur within a few hours of the receipt of the wound or may be delayed for several days. An early sign is the discharge from the wound of a foul-smelling, brownish material which later increases and contains gas; there is also local swelling of the tissues and the neighbouring skin becomes purplish. The fluid and gas extend in the subcutaneous and intermuscular tissues. When the muscles are involved they become black, friable and diffluent locally; the necrotic area of the muscle is bordered by a red zone. General symptoms are also found and are usually those of a profound toxæmia. In the late stages septicæmia may develop. The mortality rate is high (20–80 per cent.).

It must be stressed here that the isolation of clostridia from a wound does not necessarily indicate the presence of gas gangrene, the diagnosis of which must be based on clinical grounds. In many instances the organisms are merely harmless contaminants, in others they produce a local infection, termed anaerobic cellulitis, and in only few cases muscle is involved with the development of true gas gangrene. Then the condition may clinically be similar to streptococcal myositis, in which the causative organism is an anaerobic streptococcus.

Bacteriology. Gas gangrene is not a single bacterial entity as was thought by workers before the 1914–18 war; in practically all cases gross contamination with anaerobic and aerobic organisms is present. The isolation and incrimination of the main pathogenic species have consequently been problems of considerable

TABLE XXVIII

Percentage Incidence of Anaerobes in Gas Gangrene
(M.R.C. Report, 1919)

Species	Weinberg	McIntosh		Henry
	91 Cases	41 (1914–18)	52 (1918)	50 (1918)
Cl. welchii . .	77·0	44·0	67·0	80·0
Cl. œdematiens . .	34·0	—	4·0	10·0
Cl. septicum . .	13·0	19·5	16·0	16·0
Cl. sporogenes . .	27·0	36·5	39·0	—
Cl. fallax . . .	26·5	—	—	6·0

difficulty. The incidence of the various anaerobes in the diseased tissues was the subject of various investigations during the 1914-18 war (Table XXVIII.).

Various anaerobes were usually isolated, but by pathogenicity tests in laboratory animals it was found that *Cl. welchii, œdematiens* and *septicum* only were able to produce definite signs of gas gangrene. The Anaerobic Committee in their review of the disease concluded that at least three types of pathogenic anaerobic bacilli could give rise to acute gas gangrene. These were, in order of frequency, *Cl. welchii*, *Cl. septicum* and *Cl. œdematiens*. The *rôle* played by other anaerobes was not determined. It was recognized that some, such as *Cl. sporogenes*, *Cl. histolyticus* and *Cl. fallax*, were frequently present, and it was considered that these might damage the tissues in the early stages and so prepare the way for the more pathogenic species, while in the terminal stages their multiplication might be responsible for much of the proteolytic activity manifested in the infected region.

Diagnosis. The isolation of the individual organisms is extremely difficult. The material that should be submitted for examination is principally the exudate and necrotic tissue collected from the wound. In the late stages blood cultures may be positive.

The methods of examination are (1) microscopy, (2) culture, and (3) pathogenicity tests.

(1) The examination of smears, stained by Gram's method and for spores, gives some indication of the number and nature of the organisms present.

(2) Numerous cultural methods have been employed in the investigation of gas gangrene. The most valuable procedure is to add the tissue to several tubes of Robertson's meat medium, heat at 80° C. for 20 minutes to kill off the vegetative forms, subculture into meat medium, selective media (saccharolytic and proteolytic) and on to plates of solid media and incubate under anaerobic conditions.

(3) For the pathogenicity tests mice or guinea-pigs are used. In most instances normal animals are of little value owing to the presence in the inoculum of several pathogenic species. The most useful test is one in which groups of animals are used, some being previously immunized by inoculation with antitoxins of the three important pathogenic species. Five groups of mice are usually prepared: (1) normal, (2) immunized against *Cl. welchii*, (3) immunized against *Cl. septicum*, (4) immunized against *Cl.*

dematiens and (5) immunized against all three. The animals rotected against the homologous organism survive, whereas the ontrols and those protected against the other organisms are illed. In the case of a mixed infection, group 5 animals survive nd the others die; the survival time of the animals protected gainst one of the infecting organisms would, however, probably e longer than in the unprotected. This thus provides a means f isolating and identifying the individual species from the riginal mixture.

The **Nagler Reaction** is useful in identifying *Cl. welchii* and epends on the lecithinase activity of the α toxin which splits he lipo-protein complex of serum. This test can be carried out n the following manner: 20 per cent. human serum and 5 per ent. Fildes' extract are added to 6 per cent. agar at 50° C., plates re poured and dried. To the surface of one-half of a plate a few rops of *Cl. welchii* antitoxin are added and allowed to dry; naterial under examination is seeded evenly on both sides of the late; after incubation, colonies of *Cl. welchii* produce an opacity f the surrounding medium on the half of the plate without antioxin; the other half of the plate does not show this reaction wing to the presence of antitoxin and serves as a control.

The following are the criteria which are useful in the identificaon of the various *Clostridia* present in gas gangrene (see also able XXVII.):—

Cl. welchii (*B. perfringens*) is non-motile, capsulated when eshly isolated from the tissues, grows readily in glucose-agar ith much gas production, liquefies gelatin, gives rise to stormy ermentation in litmus-milk, forms an active hæmolysin; on ntramuscular inoculation into the guinea-pig gives rise to a nassive crepitant swelling containing much gas, and muscles re pale and necrotic, while the suprarenals are often congested.

Cl. septicum (*B. œdematis maligni*) is motile, grows readily in he meat medium with the production of gas, ferments glucose, naltose, lactose and salicin with the production of acid and gas. he meat is not digested, but gelatin is liquefied, forms acid and lot with some gas in litmus-milk, produces a hæmolysin, and on gar gives a filamentous growth; on intramuscular inoculation f the guinea-pig gives rise to a local hæmorrhagic, gaseous, dematous mass, the muscles are soft and deep red, while the uprarenals may be slightly congested.

Cl. œdematiens (*B. novyi*) is motile and behaves culturally as *l. septicum*; ferments glucose and maltose and produces a hæmolysin. On intramuscular inoculation of the guinea-pig it gives rise to a gelatinous mass, the local muscles are congested, purplish-red and infiltrated with small bubbles of gas, the supra-renals are not congested.

Cl. sporogenes is motile, spores readily, grows well and is markedly proteolytic, meat being digested and blackened; non-pathogenic to laboratory animals, but its presence tends to increase the infectivity of small doses of the pathogenic species.

Cl. histolyticum is motile and spores readily, actively proteolytic—digests and blackens meat with the production of tyrosin crystals, ferments glucose and maltose with the production of acid only; produces a weak exotoxin, which in large doses produces in guinea-pigs local necrosis and may even kill the animals.

Cl. fallax is motile and does not spore readily, has little proteolytic activity but ferments glucose, lactose and maltose with the production of acid and gas; when freshly isolated is pathogenic for guinea-pigs, this action is soon lost in culture.

Prophylaxis and Therapy. Two factors were mainly responsible for the greatly reduced incidence of gas gangrene after the early days of the 1914–18 war: surgical measures and the introduction of antitoxic sera. Of these, adequate and immediate surgical treatment was the more important; in all cases the wound must be cleaned and drained, while necrotic material must be removed. When the disease develops the affected tissues must be removed *en bloc;* in the severe cases amputation of the affected limb may sometimes be necessary.

SPECIFIC SERA prepared by the immunization of the horse with the various toxins, were available for general application in the late days of the 1914–18 war, and, while their value could not be definitely determined, they appeared to serve a useful purpose both in prophylaxis and therapy. In the recent war polyvalent gas-gangrene antitoxin was used extensively. All severely wounded cases received a prophylactic dose as soon as possible after wounding. The standard dose was increased threefold in the later stages and now contains: 9,000 units of *Cl. welchii* antitoxin, 4,500 units of *Cl. septicum* antitoxin and 3,000 (or 9,000) units of *Cl. œdematiens* antitoxin.

Sulphonamide and penicillin powders were also widely used in the recent war and undoubtedly played an important *rôle* in preventing the infection of wounds. There is, however, no doubt that the most important factor in the prophylaxis of gas gangrene is early and radical surgery. Antitoxin, sulphonamides and antibiotics are useful but they play a secondary *rôle* to good surgery.

In the THERAPY of the established disease, drastic measures are necessary; these include surgery, intravenous antitoxin, antibiotics, sulphonamides and blood transfusion. In spite of these, the mortality rate is high, 20–80 per cent.

Botulism

Botulism in man is a comparatively rare disease, which follows the ingestion of contaminated food, such as sausages (hence the name, *botulus* = a sausage), prepared meats, canned foods, vegetables and fish. The ætiological agent, *Cl. botulinum*, was first isolated in 1896 by v. Ermengem, who investigated a small outbreak in Belgium following the consumption of uncooked ham. From this a sporing anaerobe was isolated, and both the organism and the ham produced the same series of symptoms when fed to animals.

Cases have been extremely uncommon in this country; one outbreak at Loch Maree was reported in 1922, and a few cases were notified in 1935. At Loch Maree eight individuals developed botulism after eating potted duck paste, and all died. Outbreaks have been more frequent in America and Germany, where prepared and home-canned foods are consumed on a larger scale.

Symptoms appear a short time (18–72 hours) after the consumption of the affected food, and although some gastro-intestinal disturbance may be present they are due mainly to the action of the toxin on the central nervous system. Paralysis of accommodation, ptosis, general weakness, aphonia and dysphagia pass on, in fatal cases, to bulbar paralysis, coma and death. Sensory symptoms are seldom marked.

The mortality has varied in different outbreaks from 25 to 100 per cent., and is highest when the incubation period is shortest. On post-mortem examination there is little to be seen, the cerebral vessels are congested, and some of the nerve-cells are swollen and show signs of degeneration. The exact mode of action of the toxin is uncertain, but experimental observations suggest that the central nervous system is involved peripherally rather than centrally.

Cl. botulinum is responsible for spontaneous disease in animals, giving rise to the condition known as "limber-neck" in chickens. It has also been incriminated as the ætiological agent of a paralytic disease in cattle, but the evidence on which this relationship has been based is unconvincing.

Bacteriology. Botulism is essentially a toxæmia and not an infection. The symptoms are caused by a preformed toxin ingested with food and absorbed from the alimentary tract. It is in fact doubtful whether *Cl. botulinum* is able to multiply in the human alimentary tract.

Cl. botulinum is widely distributed in nature, being present mainly in virgin soil. It has also been isolated occasionally from samples of animal fæces, but its presence is probably due to the consumption of soil-contaminated food. Two types (A and B) were originally described, each produces a distinct toxin; type A is encountered most frequently in America and type B in Europe. Two further types (C and D) have since been isolated, but these have not been responsible for cases of human botulism. Members of the various types have been sub-divided by serological tests, *e.g.*, agglutination and complement-fixation.

Cl. botulinum grows quite well on the ordinary media under anaerobic conditions, the optimum temperature being about 30° C. Its proteolytic activities are more marked than the saccharolytic; gelatin is liquefied by all types, but the action on coagulated egg-white is variable, type A strains being the most active; acid and gas are produced in glucose, maltose and sometimes salicin (see Table XXVII.).

Cl. botulinum produces the most active bacterial toxin known; mice, guinea-pigs and monkeys are very susceptible, a dose of 0·00001 c.c. subcutaneously is sufficient to kill a mouse. Rats and dogs are very resistant.

The toxin is readily obtained by cultivation in broth (*p*H 7·4–8·0) at 35° C., the maximum amount being present on about the eleventh day. It is relatively unstable, being rapidly destroyed by light and alkalies, but it exhibits a marked resistance to weak acids; in virtue of this resistance it escapes destruction by the acids of the gastric juice; it is more resistant to heat than most other exotoxins, requiring exposure to a temperature of 80° C. for 30 minutes to effect complete destruction. A toxoid is produced by treatment with formalin.

Botulism in man invariably results from the ingestion of the preformed toxin that has been produced by the growth of the organism in food which has been contaminated either directly or indirectly with soil. Many foods, including meat and vegetable products, particularly string beans, corn, olives and spinach, are suitable media for *Cl. botulinum*, and conditions are especially favourable for growth in canned foods. The spores of *Cl. botu-*

linum are relatively resistant to the action of heat and may resist the preliminary sterilization involved in the canning process; they multiply later and produce the toxin. A temperature of 20° C. has been found suitable for growth and toxin production; growth is, however, relatively slow at this temperature. If the food is consumed in an unheated state the toxin is ingested and botulism is produced.

The food sometimes manifests some sign of contamination, there may be a rancid odour and some alteration in its physical appearance; in the case of canned foods, the cans may be blown.

Diagnosis. The clinical diagnosis may be confirmed by the demonstration of the toxin in the suspected food or by the isolation of *Cl. botulinum* from the food, vomit or fæces.

The examination of the suspected food for the presence of the toxin gives the more satisfactory results. Suspensions are made in saline and injected subcutaneously into a series of mice, some of which have been protected by the inoculation of antitoxins A and B. If the toxin is present in the suspension, the unprotected animals die.

The food is seeded into glucose-broth, incubated anaerobically at 35° C. for 10 days, filtered and injected into a similar series of mice. Cultures may be obtained by heating broth cultures at 80° C. for 30 minutes and seeding on glucose-agar plates.

Prophylaxis. Measures adopted in the prevention of botulism are (1) attention to the preparation and condition of the food; spoilage is not always detectable, but all suspected food should be discarded, and (2) adequate precaution and control in the sterilization of canned foods. Dormancy and heat-resistance are features of botulinum spores, and these must be accounted for in the process of heat sterilization. It is interesting to note that in the majority of botulism cases following the consumption of canned foods the food was home-canned and had not been subjected to the factory processes.

Therapy. Antitoxins have been prepared against A and B strains of *Cl. botulinum*, but they have not been widely used; the results of their application in the therapy of the human disease do not appear to be very satisfactory. This failure may possibly be explained by the late administration of the antitoxin in these cases.

CHAPTER XXX

MISCELLANEOUS GENERA: PSEUDOMONAS, LACTOBACILLUS AND FUSIFORMIS

PSEUDOMONAS

The following description was given by the American Committee to members of this genus: rod-shaped, short, usually motile by means of polar flagella or rarely non-motile; aerobic and facultative. Frequently gelatin liquefiers and ammonifiers; no endospores; Gram stain variable, though usually negative; fermentation of carbohydrates as a rule not active; frequently producing a water-soluble pigment which diffuses through the medium as green, blue, etc.; in some cases a non-diffusible yellow pigment is formed; many yellow species are plant parasites.

One member, *Ps. pyocyanea* or *B. pyocyaneus*, is widely distributed in nature and has a low order of pathogenicity for man, usually giving rise to local suppurative lesions.

Ps. pyocyanea is a Gram-negative, motile, non-sporing rod-shaped organism, which grows well on the ordinary media under aerobic conditions, liquefies gelatin, does not produce indole, forms acid but not gas from glucose and galactose, and produces a bluish-green pigment. This pigment has been found to consist of two substances: (1) "pyocyanin" which is bluish-green and soluble in chloroform and water; (2) "fluorescin" which is yellowish-green, insoluble in chloroform and alcohol, but soluble in water. "Pyocyanin" is only formed by *Ps. pyocyanea*, but "fluorescin" is also formed by several other organisms, including *Ps. fluorescens*. There is a definite antagonism between *Ps. pyocyanea* and *B. anthracis*, *Staphylococci* and *Streptococci* due to the production of a substance "pyocyanase" by *Ps. pyocyanea*. A hæmolysin is also formed and is distinct from the "pyocyanase."

Ps. pyocyanea is frequently present as a member of the normal flora of the skin and intestines of man. It has also been isolated for a number of conditions, of which the best known is that of "blue pus" in association with superficial abscesses, otitis media, peritonitis, empyema and bronchopneumonia; in infants it has been held responsible for cases of diarrhœa and septicæmia. *Ps.*

pyocyanea is insensitive to penicillin and is not infrequently responsible for infection of wounds receiving penicillin treatment.

In rabbits a local abscess is usually produced on subcutaneous inoculation; the guinea-pig is more susceptible, and following intraperitoneal or intravenous injection death results from toxæmia.

LACTOBACILLUS

The following description is given by Buchanan to members of this genus: rod-shaped organisms, cells frequently elongate, non-motile, without spores, Gram-positive in young cultures. Produce acid, largely lactic, from carbohydrates. When gas is produced, it is CO_2 without hydrogen. For the most part the organisms are thermophilic; micro-aerophilic.

The *Lactobacilli* have been isolated from the alimentary tract of man and animals, carious teeth and milk which has undergone fermentation. *L. acidophilus* and *L. bifidus* are frequently present in the fæces of breast-fed infants. Doderlein (1892) isolated a member from the acid vaginal secretion of pregnant women; this was termed Doderlein's bacillus, but it is now accepted as a strain of *L. acidophilus.* Another member, *L. caucasicus*, was originally isolated from "kefir", the fermented milk of the Caucasus, while *L. bulgaricus* was obtained from the fermented milk of Bulgaria. *L. odontolyticus* was recovered from carious teeth, and the Boas-Oppler bacillus was observed by Oppler (1895) in the stomach contents of an individual suffering from a gastric carcinoma.

These organisms are Gram-positive, slender, non-motile and non-sporing rods, which may exhibit pleomorphism. Growth is never profuse on the usual media, but is increased by enrichment with glucose and blood. On first isolation they do not, as a rule, grow under aerobic conditions; micro-aerophilic or strictly anaerobic environment may then be necessary, but after a few subcultures most species grow aerobically. The optimum reaction of the medium is about pH 7·0.

The *Lactobacilli* behave in most respects as the non-sporing organisms to bactericidal agents, being readily killed by heat, but an important distinction is their high resistance to acids. For this reason they are referred to as "acidophilic" or "aciduric" bacilli. This resistance is employed in the isolation of these organisms from contaminated material. A commonly employed method is to incubate the material in 0·5 per cent.

acetic acid broth for 1–3 days and then subculture on 2 per cent. glucose-agar.

Little proteolytic activity is displayed, indole is not formed and gelatin is not liquefied. The saccharolysis is more marked, though variable; acid is usually produced in glucose, maltose, lactose and sucrose. Litmus-milk is acidified and frequently clotted.

These organisms appear to have no pathogenic action in man or animals. They are normal members of the intestinal flora and are particularly numerous in individuals fed on a carbohydrate diet.

Living cultures, particularly of *L. acidophilus*, administered by the mouth have been recommended by some workers, particularly in America, for various intestinal disorders, *e.g.*, to suppress the growth of putrefactive organisms and to relieve constipation and the so-called "auto-intoxication" of intestinal origin. The results have, however, been far from satisfactory. This may be due to the use of unsatisfactory preparations, as it has been found that only certain strains of *L. acidophilus* are able to multiply in the human intestines. *L. bulgaricus*, which has been used therapeutically in this respect, is unable to establish itself in human intestines.

FUSIFORMIS

This genus has been defined in the following manner: obligate parasites; anaerobic or micro-aerophilic; cells frequently elongated and fusiform, staining somewhat unevenly. Filaments sometimes formed; non-branching. Non-motile. No spores. Reaction to Gram variable. Growth in laboratory media feeble.

This group has received relatively little attention; it is considered by some workers to be related to the *Corynebacterium* and *Pfeifferella* groups. While some members belong to the normal bacterial flora of the alimentary tract, others have been isolated from ulcerative conditions of the throat and various suppurative lesions, including appendicitis. One member, *F. fusiformis*, is frequently associated with a spirochæte in the lesions of Vincent's angina, but its *rôle* in the causation of this condition is uncertain.

These organisms tend to be large fusiform rods, but pleomorphism is a common feature and branching may occur. Many are Gram-positive. Growth occurs quite readily at 37° C. under anaerobic or micro-aerophilic conditions. In some cases there appears to be little doubt that they are either wholly or partially responsible for the lesions from which they have been isolated. In others they are either harmless parasites or secondary invaders. Necrotic lesions and death have been produced experimentally by some members in laboratory animals.

Fusiformis organisms are considered to be responsible for the condition termed " necrobacillosis ". This condition is usually fatal and consists essentially of multiple abscesses, frequently found along the pleural margins of the lungs and associated with a foul-smelling empyema. The organisms may be present in large numbers in the pus or blood-stream but they are difficult to culture ; anaerobic conditions are essential. They are Gram-negative and non-sporing and, while they become very pleomorphic on culture, they do not present a fusiform appearance ; in consequence *F. necrophorus* does not seem an appropriate title for these organisms.

LISTERELLA

Listerella organisms are Gram-positive, non-sporing rods ; cocco-bacillary forms are usual although filaments may occur. They are motile ; motility is, however, most satisfactorily demonstrated by incubation at 26° C. Growth occurs on the usual laboratory media under aerobic conditions. The organisms are pathogenic to many animals including rabbits, guinea-pigs and mice, and tend to produce a monocytosis in the blood ; as a result they have been termed *Listerella monocytogenes.* A few instances of isolation from human lesions such as meningitis, septicæmia and perhaps infectious mononucleosis, have been reported. The ætiological relation of these organisms to the various conditions has, however, not been definitely established.

The taxonomic position of the *Listerella* organisms has not been determined. They appear to have points of similarity with members of the *Erysipelothrix* group, but there are several significant differences.

CHAPTER XXXI

SPIROCHÆTES : SYPHILIS, YAWS, WEIL'S DISEASE, SEVEN-DAY FEVER, RELAPSING FEVER AND VINCENT'S ANGINA

THE spirochætes possess affinities with both protozoa and bacteria. In consequence their biological status is uncertain and their study has been neglected by both protozoologists and bacteriologists. It is therefore not surprising that our knowledge of these organisms is far from complete.

The term *Spirochæte* was first used by v. Ehrenberg (1833) to indicate certain elongated, spiral, motile organisms present in stagnant water. Since then many varieties have been described and *Spirochæte* is now generally employed to indicate all pathogenic and non-pathogenic spiral organisms which exhibit motility without possessing flagella.

General Properties. The *Spirochætes* possess a spiral, flexible form and present some morphological resemblance to members of the *Spirillum* group, which are, however, rigid and have flagella.

A characteristic feature of the spirochætes is that they are motile and yet have no flagella. The motility is produced by means of flexion of the body, by rotation around the long axis, *i.e.*, corkscrew motion, or by a general movement of the protoplasm termed translation. Movement takes place equally well in a forward or backward direction, *i.e.*, there is no antero-posterior polarity.

There is considerable variation not only in the size of the different spirochætes, but also in the number and regularity of the spirals. Most spirochætes do not stain readily and special methods are required for their demonstration in stained preparations. They are therefore frequently examined in an unstained condition, but as the refractility of their protoplasm is low the ordinary hanging-drop technique is of little value and dark-ground illumination is required.

The method of multiplication is by transverse division only ; there is no sexual phase of reproduction. The spirochætes, however, do not grow readily *in vitro*, and the ordinary media are of little value for cultural purposes. Noguchi (1911) introduced a satisfactory medium, composed of sterile ascitic fluid arranged in long narrow tubes and containing a piece of sterile rabbit-kidney. This is particularly useful for the cultivation of the anaerobic members and may be solidified by the addition of agar for the isolation of spirochætes from contaminated material. For the more aerobic spirochætes, such as the blood spirochætes and *Lept. icterohæmorrhagiæ*, success has followed the use of semi-solid agar to which fresh rabbit blood is added immediately before use. In many cases isolation from the tissues is extremely difficult, but when growth occurs subcultures are usually obtained without much trouble.

The resistance offered to destructive agents is similar to that exhibited by the vegetative bacteria. Most spirochætes are killed by temperatures over 50° C., but can withstand low temperatures. They are very susceptible to drying ; this usually accounts for the early death of the pathogenic members outside the animal body.

The serology is similar to that of the bacteria ; agglutinins, spirochæticidins and protective bodies have all been demonstrated. A so-called " adhesion phenomenon ", the *Rieckenberg reaction*, is also found ; in the presence of the specific antiserum the spirochætes are altered in such a way that small particles, such as platelets and bacteria, adhere to them.

The spirochætes have been loosely divided into three groups : (*a*) pathogenic, (*b*) commensal, and (*c*) the free-living. The free-living types are found mainly in fresh- and sea-water containing organic matter. A large number of spirochætes inhabit the bodies of man and animals as commensals. It appears probable that a number may become pathogenic and be responsible for certain ulcerations of the alimentary tract. Some pathogenic members have been isolated from stagnant water and may also exist as commensals ; others, however, are strict parasites. There is thus much overlapping of the various groups.

Various classifications have been suggested, all of which are based mainly on morphological criteria and are therefore open to question. As our knowledge of the spirochætes is so incomplete a final method of classification is not yet available. A useful and convenient division, which is now widely used, is one in which four main groups are included :—

(1) **Spirochæta**—free-living members with an axial fibre and an unsegmented protoplasm ; non-pathogenic to higher animals.

(2) **Cristispira**—free-living members with a band-like membrane

or crista running spirally along the length of the body ; the body is divided by transverse septa ; non-pathogenic to higher animals.

(3) **Treponema** (including the spironema class)—members with a number of primary spirals, an indefinite axial fibre and sharply pointed extremities ; some are pathogenic, *e.g.*, *Trep. pallidum*, the causative agent of syphilis.

(4) **Leptospira**—members with closely set, regular, primary spirals, and an indefinite axial filament ; the ends are very flexible and are frequently arranged at angles to the main axis ; some are pathogenic, *e.g.*, *Lept. icterohæmorrhagiæ*, the causative agent of Weil's disease.

The main diseases of man produced by the spirochætes are :—

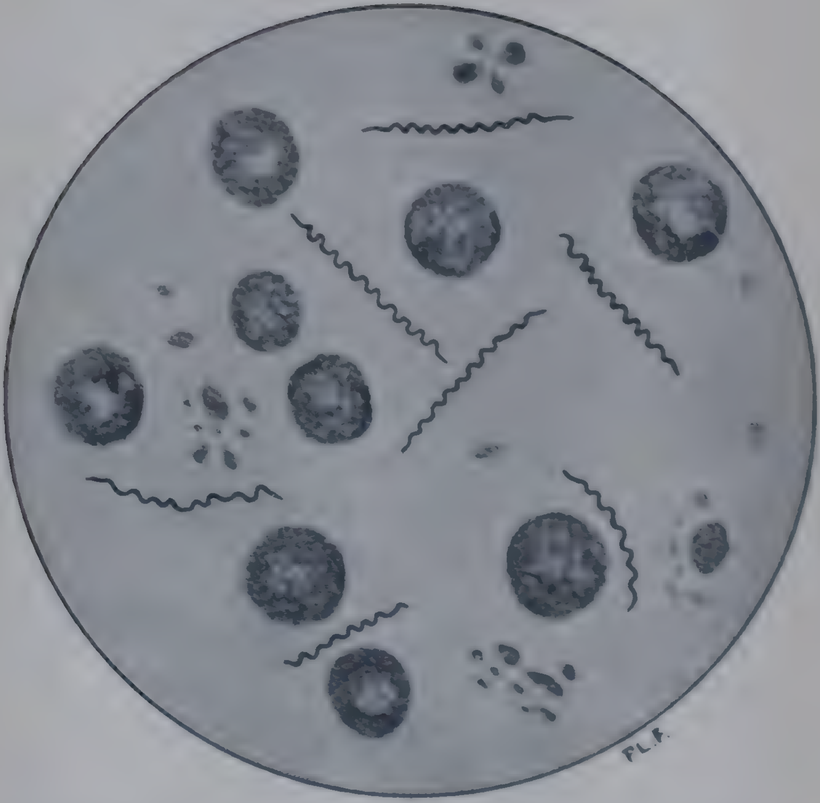
Syphilis . . .	*Trep. pallidum.*
Yaws . . .	*Trep. pertenue.*
Relapsing fever . .	*Trep. recurrentis.*
Weil's disease . .	*Lept. icterohæmorrhagiæ.*
Vincent's angina . .	*Trep. vincenti.*

Syphilis

Syphilis is a disease of some antiquity. The origin of the disease is uncertain, but it appears probable that it was introduced into Europe by Columbus and his crew on their return from discovering the New World. There are several records of the alarming and widespread outbreaks of the disease in Europe during the last decade of the fifteenth century. The disease then presented a clinical picture quite different from that seen at the present time. It ran an acute course with symptoms of marked severity and a high mortality rate in the early stages. This suggests the introduction of a pathogenic organism into a virgin soil. The name " Syphilis " originated at this time as a result of a poem by Fracastor in which the hero, a shepherd named " Syphilis ", was smitten with the disease for disrespect shown to the Gods.

Although many reports of the condition had been published in the meantime, the ætiological agent was not detected until 1905. Then Schaudinn and Hoffmann discovered a spiral organism in fluid from chancres and the associated enlarged inguinal glands. Lesions had, however, been previously produced in the rabbit by Haensell (1881), and in apes by Metchnikoff and Roux (1903). Following the demonstration of the ætiological agent, then

PLATE IV



Trep. pallidum in tissue (stained by Levaditi's method). × 1000.

[*To face p.* 371.

termed "Spirochæta Pallida" and now known as *Treponema pallidum*, much progress was made in the study of the disease. Wassermann and his colleagues introduced the serological reaction, universally known as the "Wassermann test," in 1906; Ehrlich (1910) published his results of treatment by salvarsan; and in 1913 Noguchi cultivated the organism from the brain of cases of general paralysis.

Bacteriology. *Trep. pallidum* is approximately 8–14μ in length and contains some 10 regular spirals, the depth and length of each being about 1μ. The spirals tend to be more open and shallower at the ends, which terminate as fine, tapering, pointed threads (see Plate IV.). Division takes place by transverse fission. Motility is sluggish if present.

It has been suggested that there is a granular, filterable phase in the life cycle of the organism, but this view is not upheld by filtration tests. Further developments are required before this theory can be accepted.

Trep. pallidum is difficult to stain, and is examined most satisfactorily either unstained by dark-ground illumination or by methods which stain the background of the smear but leave the organism unstained, *e.g.*, mixing with Indian-ink or Congo-red, spreading and drying. For stained smears, Giemsa's method, which is prolonged for 16–24 hours and colours the organisms pinkish, or special silver methods such as Fontana's, by which the organism is stained black, are used. For sections Levaditi's silver method gives good results (Plate IV.).

Cultivation is not easy and there is still some doubt whether *Trep. pallidum* has actually been cultivated *in vitro*. Spirochætes morphologically resembling *Trep. pallidum* have been isolated in pure culture from syphilitic lesions, but these have generally proved avirulent on injection into rabbits and monkeys. Anaerobic conditions and an enriched medium containing serum or ascitic fluid are necessary, and growth occurs most favourably at 37° C. and with a reaction of pH 7·2–7·5; subcultures should be made every 10–14 days.

Trep. pallidum is readily killed by bactericidal agents and is particularly susceptible to drying.

Experimental infection can be produced in monkeys, rabbits and mice by intratesticular, corneal or intradermal inoculation of tissue containing the spirochætes. The rabbit is the animal of choice, as infection is readily obtained and its course is comparable to that found in the human disease. On intratesticular inoculation, after an incubation period of 2–3 weeks, the testicles become swollen and indurated, the inguinal glands are later involved and a generalized infection follows, *i.e.*, there is a stage analogous to the secondary stage in man. It is, however, important to note that another allied treponema, *Trep. cuniculi*, gives rise to spontaneous lesions in rabbits and, as it is morphologically similar to *Trep. pallidum*, the failure to differentiate these organisms may be a source of irregularities in experimental work.

Pathogenesis. Syphilis in man may be either congenital or acquired.

Congenital syphilis results from syphilis in the mother. The organisms pass from the maternal to the fœtal blood by way of the placenta. A syphilitic mother does not necessarily produce a syphilitic infant, as if she is infected very late in pregnancy, or if the disease has reached the latent stage, a healthy infant may result. The explanation is that at these particular stages of the disease the organisms are not usually present in the blood-stream and consequently have not the opportunity of reaching the fœtus. It is doubtful whether transmission from the father occurs other than by producing infection in the mother primarily and in the fœtus secondarily. In congenital syphilis the infection is generalized and the organisms are widely disseminated ; lesions may be present in most tissues, particularly the liver, spleen and bones. Death of the fœtus not infrequently occurs *in utero.*

In **acquired syphilis** the organisms gain entrance as a rule through the mucous membrane of the urino-genital tract ; in some cases the primary infection may occur in the mucosa of either the mouth or the skin. In a few instances infection has followed blood-transfusion, in which event the organisms are implanted directly into the blood-stream.

Syphilis is usually transmitted by direct contact during sexual intercourse. It may also be contracted either directly as a result of kissing, wet-nursing and circumcision, or indirectly through the agency of towels, handkerchiefs, pipes, etc. As the life of *Trep. pallidum* outside the body tissues is probably short, cases of indirect transmission are relatively few.

Following the entry of the organisms into the tissues, there is usually an incubation period of some 3 weeks, but this may vary from 10–90 days. The course of the disease is characterized by its chronicity and is divided clinically into three stages—primary, secondary and tertiary, which are not clearly demarcated.

The "primary stage" consists initially of the appearance of a local lesion or chancre at the site of infection. It is usually single, painless, indurated and tends to ulcerate. When the chancre is well developed there is an associated enlargement of the regional glands.

The "secondary stage" sets in some 6–12 weeks after the appearance of the primary lesion and represents a generalization of the infection. The chief symptoms are sore-throat, cutaneous rash, joint and bone pains, headache, general enlargement of the lymph glands and eye lesions with some pyrexia during the initial phase. This stage may last from a few months to several years and merges imperceptibly into the tertiary stage. In some cases the lesions are so mild that they may escape notice.

The "tertiary stage" is usually observed after a so-called "latent period", in which the infection has localized in some organ where it has been slowly developing. It is manifested by the appearance in any tissue of chronic, progressive and destructive lesions or gummata, which are of a granulomatous nature. When healing occurs marked scarring is produced. This stage lasts indefinitely. In the late phases changes in the nervous system, such as tabes dorsalis or general paralysis, may appear. These late changes are considered by some people to represent a "fourth or a quaternary stage". This is the most disabling stage of the disease, and frequently gives rise to insanity or premature death.

Observations made in both the human and the experimental disease indicate that, while the disease remains localized clinically during most of the primary stage, the infection actually soon becomes generalized. In the very early stages the organisms can be detected only in the local lesion, but in the later part they can also be obtained from the regional glands and the blood-stream. In the secondary stage the organisms are widely disseminated, being present in the blood-stream, mucous patches of the throat, rash, semen and lymph glands. As this stage subsides the organisms become increasingly difficult to demonstrate and, in the tertiary stage, they tend to remain localized in the specific lesions. They occasionally generalize and have been isolated from the blood-stream during the late stages of the disease.

It is thus important to note that individuals are most infectious during the early stages of the disease when the lesions are relatively mild and may escape notice. In the late stages, when the lesions attract attention, the infectivity of the individual is relatively low. These are points of great importance in the control of the disease.

Immunity. Man has little natural immunity to syphilis, although the marked change in the clinical picture of present-day syphilis from that seen in the Middle Ages suggests that a certain degree of general resistance has developed in man. Nevertheless, all uninfected individuals are susceptible to infection. There is, however, much evidence to indicate that during the course of the disease some immunity of a low grade is acquired against re-infection. There is thus an "infection-immunity" as found in tuberculosis, but while there appears to be increased resistance to re-infection, spontaneous cure of the primary infection does not take place. It has been found in rabbits that with the development of a well-marked primary lesion super-infection of the animal with *Trep. pallidum* becomes increasingly difficult. Infected rabbits, successfully treated in the early stages of the disease with arsenical preparations, can later be re-infected without difficulty, whereas animals treated in the later stages are usually resistant. In man the rarity of re-infection during the course of the disease is well known. It is, however, important to note that the intradermal inoculation of the mouse gives rise to infection without the production of a primary lesion and the spirochætes have been demonstrated in various tissues, *e.g.*, liver and spleen, several months after the original injection. If such a phenomenon followed the re-inoculation of infected rabbits or man, re-infection would be extremely difficult to detect. It is considered by many workers that the termination of the secondary stage, with the great reduction in the numbers of the organisms in the tissues, is brought about by some form of immunity response by the host. The search for antibodies has, however, yielded little information about the nature of this response in syphilis.

Some workers have suggested that there are two distinct strains of *Trep. pallidum*—one being neurotropic and the other dermotropic. It has been observed that, when the dermal manifestations of the early stages are well marked, neurosyphilis does not result; whereas nervous disorders are found in cases in which the early superficial lesions have been slight. The occurrence of nervous lesions in individuals infected from the same source has also been reported. These observations are, however, more than counterbalanced by many negative observations, *e.g.*, neurosyphilis is rare in coloured people, but white people infected by them frequently develop nervous lesions.

Further evidence is therefore required before the existence of the two strains can be accepted.

Diagnosis. The bacteriological methods used in the diagnosis of syphilis are (1) microscopy and (2) serology.

(1) Microscopy is the most satisfactory method in the early stages of the disease. The organisms can usually be seen without difficulty in the exudate of the primary or secondary lesions. In the collection of the exudate the surface of the lesion should be cleaned to remove contaminants and the serous fluid obtained; hæmorrhage should be avoided, as the corpuscles interfere with the microscopical examination. Closed lesions should be punctured and the regional glands aspirated.

The examination is made either under dark-ground illumination or on stained preparations. Under dark-ground illumination *Trep. pallidum* presents a silvery appearance with regular spirals and pointed ends and may exhibit rather sluggish motility. Other spirochætes may be present, particularly if the specimen has been collected from the mouth, and some difficulty may be experienced in identifying *Trep. pallidum.*

Specimens may be stained by Giemsa's or Fontana's method, but a more satisfactory procedure is to mix the exudate with indian-ink, prepare smears and dry. *Trep. pallidum* stands out unstained against a dark background.

(2) Serological tests are of little value in the early stages of the disease, but after the development of the secondary stage they have proved of great clinical importance. Two main tests have been used extensively in the sero-diagnosis of syphilis—complement-fixation (Wassermann test) and precipitation (Kahn test) (see Chapter XXXVIII).

Wassermann Test. The rationale of this test may more easily be understood by a preliminary recapitulation of the essential features of the complement-fixation test. Certain antigens in the presence of the homologous antiserum are able to fix complement; red blood corpuscles (R.B.C.) in the presence of the inactivated homologous antiserum are unchanged, but when complement is also added lysis takes place. Sensitized R.B.C. consequently constitute a satisfactory indicator for the presence of complement. Antigen, inactivated patient's serum and complement are incubated at 37° C. for 1 hour, sensitized R.B.C. are then added, the mixture is re-incubated for a further hour and the presence or absence of lysis determined; the reaction is represented diagrammatically in Table XXIX.

TABLE XXIX

(1) **Positive reaction.**
Antigen + serum
+ complement
+
sensitized cells = **no lysis,** *i.e.*, fixation of complement.

(2) **Negative reaction.**
Antigen + serum
+ complement
+
sensitized cells = **lysis,** *i.e.*, no fixation.

While the above principles hold for the Wassermann test, it is important to note that the reaction is not specific in the immunological sense. When Wassermann and his colleagues (1906) first applied complement fixation to the diagnosis of syphilis, they employed as antigen a suspension of a syphilitic liver. It was, however, later found that satisfactory results were obtained by the use of an extract of normal liver, which naturally did not contain *Trep. pallidum.* Further observations indicated that alcoholic extracts made better " antigens " than the watery extracts and that the reacting substance was contained in the lecithin fraction of the lipoids and was soluble in alcohol. Some time later it was shown that the " antigen " could be rendered more sensitive by the addition of an alcoholic solution of cholesterol. The " antigen " in general use at the present time is prepared by adding a 1 per cent. alcoholic solution of cholesterol to an alcoholic extract of normal human heart muscle and diluting rapidly with saline to give a turbid suspension.

The antigen is thus non-specific, but the test exhibits a high degree of disease-specificity. The explanation of this is not known. The nature of the reacting substance in the serum has not been determined ; it is uncertain whether it is a true antibody or a specifically changed property of the serum. The reacting substance may be a tissue disintegration product resulting from the multiplication and activity of *Trep. pallidum* or it may be a true antibody stimulated by the presence of these disintegration products. Another theory suggests an analogy to the stimulation of the heterophile antibodies.

The original test has been modified by many workers, and at the present time it is improbable that the exact technique of any two laboratories is identical. It is usual to carry out a quantitative as well as a qualitative test. In order to do this it is possible to vary the quantities of either antigen, complement or patient's serum. In practice the patient's serum is generally varied and the other factors standardized.

Despite the limitations of our knowledge concerning the mechanism of the reaction, in the hands of experienced workers the results are extremely reliable. The test has consequently been one of the most widely used of the serological diagnostic reactions. In the primary stage of syphilis the reaction is frequently negative. With the development of the secondary stage, and in the absence of treatment, a positive result is obtained in 90–100 per cent. of cases. A high percentage of positive results is also obtained when active lesions are present in the tertiary stage, but negative results may be given during the so-called " quiescent " or " latent " period. In cases of neurosyphilis the reaction is usually given by both serum and cerebrospinal fluid. It is, however, accepted that the reactions given by the serum and the fluid may not run parallel; this is particularly so in treated cases.

Cases rigorously treated in the early stages may never give a positive Wassermann reaction. It thus follows that while a positive diagnosis is strongly indicative of the presence of syphilis, a negative test does not exclude it. A persistent negative reaction in a suspected case of secondary syphilis would, however, almost certainly exclude the disease. In all instances it is essential to correlate the clinical and laboratory evidence before making a diagnosis.

It is sometimes stated that a positive Wassermann reaction is given by the serum of patients in the acute stages of scarlet fever or suffering from tuberculosis. This appears to be incorrect. When positive reactions have been reported in these conditions there has most probably been a superadded syphilitic infection; the majority of such cases give a negative reaction.

Precipitation Tests. Many flocculation tests have been introduced for the diagnosis of syphilis: Sachs-Georgi, Sigma, Kahn, Meinicke, etc. Of these, the Kahn test is used most widely at the present time. In all two reagents only are required. This is an obvious advantage over the complement-fixation test.

Kahn Test. This is used extensively in America. The antigen consists of an alcoholic extract of the ether-insoluble lipoids of beef heart. The inactivated serum is added to the antigen-suspension and the mixture is shaken vigorously for 3 minutes. After standing for 3 minutes saline is added and readings taken ; the presence of precipitation indicates a positive reaction.

The test is probably as reliable and sensitive as the Wassermann reaction, and, as it is simpler and quicker to carry out, the latter test has been superseded in some laboratories ; the main disadvantage of the Kahn test is that doubtful results are sometimes difficult to read. It has been recommended that both complement-fixation and flocculation should be used in the sero-diagnosis of syphilis. Although the two tests agree in a high percentage of cases, there is not complete overlapping and it is possible for cases to be missed if only one test is used. In practice the use of both tests is not always possible, but it is certain that, when unexpected or unsatisfactory results are obtained by one method, the other should always be tried.

Prophylaxis. There are no specific measures of prophylaxis. The prevention of syphilis is an important and difficult problem of public health administration. This is indicated by the fact that, in spite of the various measures adopted to control the disease, some 19,000 new cases were reported in England and Wales during 1935. Such questions as the regulation of prostitution, education of the public and the compulsory notification of the disease have been advocated, but a unanimous opinion has not been obtained. There has been a big increase in the incidence of syphilis during recent years as a result of war-time conditions and consequent moral relaxation. *Trep. pallidum* is harboured almost exclusively by the actual cases of the disease, early and adequate treatment of which would naturally decrease the chances of dissemination of the spirochætes.

Therapy. Arsenical therapy, introduced by Ehrlich (1910), has given excellent results in the early stages of the disease, but it is necessary to enforce prolonged courses of treatment. In the later stages the results are less satisfactory. Salts of mercury and bismuth are also valuable therapeutic agents, and in the later stages potassium iodide may prove useful. In neurosyphilis, particularly general paralysis, malarial infection has recently been introduced and beneficial effects have been reported. These appear to be mainly the result of the induced pyrexia.

It is important to note that the rendering of a positive serum-reaction negative by treatment does not necessarily indicate a cure; treatment should be continued until all signs of the disease have cleared up and the serological tests remain persistently negative. A complete cure is extremely difficult to determine. There is, however, no doubt that syphilis is curable if proper treatment is carried out in the early stages of the disease.

Treatment with penicillin has been widely practised during the past two years. Large doses are essential and the usual course has been 2,400,000 units given in doses of 40,000 units three-hourly for seven and a half days. It is too early for a final assessment of the results but, although the immediate response of early cases is good, some relapses (in some trials up to 15 per cent.) have been reported. In consequence it is now recommended that either combined treatment with penicillin and arsenoxide or an increased dosage of penicillin should be given.

Yaws

Yaws is a tropical disease found mainly in the children and young adults of rural districts. The lesions are found on the skin, and consist of papules which develop into an encrusted granulomatous eruption; apart from the spleen, the internal organs are not generally affected. Yaws is not usually a venereal disease; it is transmitted by direct contact, the organism gaining entrance by open wounds or slight abrasions.

The causative organism is a spirochæte, *Trep. pertenue*, first described by Castellani in 1905. It is morphologically indistinguishable from *Trep. pallidum*, and it has been suggested that the two organisms are related, *i.e.*, yaws is a modified form of syphilis. Conclusive evidence of this relationship has not, however, been produced. It is well known that patients suffering from yaws give a positive Wassermann reaction, but cross-immunity tests indicate that the organisms are not absolutely identical. Patients with yaws have developed syphilis and *vice versâ*. Experiments in monkeys have yielded the same results; a definite immunity was not produced by one organism against the other. It has, nevertheless, been noted that syphilis is uncommon in individuals who have suffered from yaws in childhood, *e.g.*, syphilis is rare in the Fiji Isles where yaws is rife. Further work is required before the relationship between yaws and syphilis can be definitely determined.

Arsenical preparations and bismuth salts have proved useful therapeutic agents.

Weil's Disease or Infectious Jaundice

Weil's disease is a widespread disease occurring mainly in sewage workers, miners, agricultural workers in damp regions, *e.g.*, rice-fields of Japan, and in soldiers during war-time conditions; it may, however, occur in any individual coming in contact with stagnant water in rat-infested districts. The disease appears to be relatively common in this country; several cases have been reported among sewage workers in London, while an outbreak at Aberdeen was recorded in 1934 among fish cleaners working in rat-infested buildings. Further investigations by Smith and Davidson have shown that the condition is particularly prevalent among the fish workers in Aberdeen; serological tests were carried out on 210 workers in the fish-cleaning trade, and some 25 per cent. gave a positive result, indicating previous infection. A large number of controls were tested and all gave a negative result. The ætiological agent, *Lept. icterohæmorrhagiæ*, was first discovered in cases of the disease by Inada and his colleagues in Japan during 1915.

A few cases have been reported of human infection with the allied spirochætes, *Lept. canicola* and *Lept. grippo-typhosa*; *Lept. canicola* is primarily a pathogen in dogs, giving rise to nephritis leading to uræmia. *Lept. grippo-typhosa* is a pathogen of rodents and was responsible for a few cases of Weil's disease during the recent Normandy campaign. In man the clinical signs of infection with these organisms are similar to those found in infection with *Lept. icterohæmorrhagiæ*.

Bacteriology. *Lept. icterohæmorrhagiæ* varies in length, the average being 7–14μ, and contains a number of closely wound spirals, each about 0·25–0·5μ in length. It has tapering ends which are frequently set at an angle to the main axis, giving to the organism the form of the letter C, L or S. It exhibits active motility.

Growth is obtained without much difficulty on artificial media, such as semi-solid blood-agar. Multiplication occurs at or near the surface in 6–8 days when incubated at a temperature of 30° C. Cultures can be kept at room-temperature for 4–6 weeks.

Transmission. *Lept. icterohæmorrhagiæ* has not only been isolated from cases of Weil's disease, but it has also been established that the organism is a frequent pathogen of rats, by which it is commonly excreted in the urine. The number of infected rats, *R. norvegicus* (the grey sewer rat), caught during examinations of the wild-rat population in different countries has varied from 10 to 30 per cent. The disease in the rat is comparatively mild, jaundice is not found. Another interesting point is that *Lept. icterohæmorrhagiæ* can survive in stagnant water, under favourable conditions, for several months.

These observations indicate the probable mode of transmission of the infection in man, which is generally considered to take the following course. The disease is primarily one of rodents, particularly the grey rat, which excretes the organism in the urine. In this way the organism is widely disseminated, and in badly drained, rat-infested areas stagnant water becomes readily contaminated. Individuals working in these regions become infected as a result of the contaminated material coming in contact with cuts, scratches or minute abrasions on the exposed skin, the nasal mucosa or conjunctiva.

After an incubation period of 5–7 days, there is an initial febrile stage of some 7 days, during which the main signs are headache, prostration and muscular pains, with marked irregularity of the temperature. The condition may then settle down; in a minority of cases it passes on to an icteric stage, which usually lasts from 7–13 days. There is either a gradual subsidence of the jaundice with the commencement of convalescence or death supervenes as a result of hæmorrhage. The mortality rate among the diagnosed cases has varied in different outbreaks from 6–32 per cent.

It is important to note that the appearance of jaundice is not an essential feature of the disease, and that in exposed workers many influenza-like illnesses are probably caused by a leptospiral infection. This is strongly suggested by the findings of Smith and Davidson (1936), who consider that infection with *Lept. icterohæmorrhagiæ* occurs in three grades: (*a*) severe infection with jaundice, (*b*) mild pyrexial infection without jaundice, and (*c*) latent infection without clinical manifestations.

The condition can be reproduced experimentally in guinea-pigs, which are highly susceptible. After intraperitoneal infection of the organism the main symptoms are fever and jaundice; the temperature falls about the fourth day when the jaundice appears; the disease terminates in death after 5–10 days. Infection may also be obtained by rubbing infected material on the skin or conjunctiva. On autopsy there is generalized jaundice with multiple hæmorrhages and enlargement of the spleen; the spirochætes are most numerous in the liver. Other animals are relatively resistant.

Diagnosis. The distribution of the organisms in the body depends on the stage of the disease; during the first stage they are widely distributed and can be readily demonstrated in the blood-stream. After 7 or 9 days they become increasingly difficult to find in the blood, but can be demonstrated in the urine, in which they are most numerous about the third or fourth week They then gradually disappear and are rarely found after the fifth week. In individuals dying during the first week spirochætes can be found in many tissues, afterwards they are most numerous in the kidney.

The bacteriological methods employed in the diagnosis of Weil's disease are (*a*) microscopy, (*b*) pathogenicity tests, and (*c*) serology.

(*a*) Microscopical examination is usually carried out on unstained material under the dark-ground illumination. The spirochætes can be demonstrated in the blood during the first week, but the most satisfactory results are obtained with the urine after the initial stage of the infection. Attention is directed to the presence of the closely wound spirals and hooked ends, but atypical forms are frequently seen, particularly with urine having a strongly acid reaction.

Tissues collected at autopsy are most satisfactorily stained by Levaditi's method.

(*b*) Pathogenicity tests are carried out in the guinea-pig. During the first week blood and during the later stages the urine are injected subcutaneously or intraperitoneally; 2–5 c.c. are injected, and if the spirochætes are present a typical infection is produced.

(*c*) Various serological tests have been devised—agglutination, complement-fixation, lysis, neutralization and the demonstration of the adhesion phenomenon. An agglutination test introduced by Schüffner has recently been used with success; dilutions of the patient's serum and a suspension of the leptospira are mixed in a small sterile container and incubated at 32° C. for 3 hours. A drop of the mixture is then examined by dark-ground illumination for lysis and agglutination of the leptospira. Agglutination is usually seen in the lower and lysis in the higher dilutions of the serum. The responsible antibodies have been demonstrated after the seventh day of the illness. This method of examination is particularly valuable in those cases settling down after the febrile stage and not manifesting jaundice. A macroscopic agglutination test, in which young actively growing cultures of leptospiræ are used, has since been tried with success. The mixture of serum and culture is incubated for 3 hours at 37° C., followed by 30 minutes at 55° C. The results are read with a hand lens.

If negative results are obtained in suspected cases similar tests should be carried out with *Lept. canicola* and *Lept. grippo-typhosa*, which have antigenic differences from *Lept. icterohæmorrhagiæ*.

The identification of *Lept. icterohæmorrhagiæ* in samples of stagnant water, slime, etc., is more difficult, as many closely allied but non-pathogenic forms may also be present in these specimens. Isolation is most readily carried out by the injection of guinea-pigs, in which *Lept. icterohæmorrhagiæ* produces the characteristic infection.

Prophylaxis. Important measures in the prevention of Weil's disease are the suppression of rats, attention to badly drained areas and the treatment and protection of wounds, scratches, etc. in individuals likely to be exposed to infection.

Therapy. Arsenical compounds have proved unsatisfactory as a therapeutic measure, but favourable results have been reported following the use of immune horse-serum. Penicillin in large doses appears to give satisfactory results.

Seven-day Fever of Japan

A febrile condition lasting about a week is encountered in Japan chiefly in agricultural workers; jaundice is uncommon, and the death-rate is nil. The ætiological agent, *Lept. hebdomadis*, is closely related to *Lept. icterohæmorrhagiæ*, from which it can be distinguished by serological tests. It is present in the blood during the first week of the illness, after which it passes to the kidneys and can usually be obtained from the urine from the eighth to the fortieth day after the onset. The organism is pathogenic to young guinea-pigs, producing enlarged glands, slight jaundice and a mortality rate of about 60 per cent. The vector of the organism appears to be the field-mouse.

Spirochætes have also been isolated from an allied febrile condition in Japan known as "autumnal fever". Two serological types have been distinguished—one is identical with *Lept. hebdomadis*, the other is more virulent and has been termed *Lept. autumnalis*.

Relapsing Fever

Relapsing fever is the name given to a group of closely related diseases which are widespread in tropical and sub-tropical regions. The disease is characterized by the sudden onset, severe frontal headache, fever and muscular pains. The incubation period may vary from 3–10 days, and the fever generally persists for some 5 days, when it falls by crisis. After remaining normal for about a week there is a recurrence of the symptoms ; there may be as many as ten relapses before the condition finally settles down. The mortality rate is usually low, about 5 per cent. In fatal cases the post-mortem findings are not particularly characteristic.

The ætiological agent of European relapsing fever was first observed by Obermeier (1873) in the early days of bacteriology, during an outbreak in Berlin. He considered the organism to be a spirochæte ; this is now termed *Trep. recurrentis*, and is responsible for European relapsing fever. Subsequently a number of similar but serologically distinct organisms have been described in outbreaks of relapsing fever in different countries. These are considered to be distinct species and have been given separate names, such as *Trep. carteri* of the Indian disease, *Trep. duttoni* of the African, *Trep. novyi* of the American, and others.

All present a similar morphological picture. The average length is about 14μ, and each organism contains some five to seven loose spirals, which have a wave-length of 2–3μ. The ends are pointed and motility is active. *Trep. recurrentis* were first cultivated by Noguchi (1912) in ascitic fluid with a small piece of fresh rabbit kidney. Growth takes place most readily at 30° C. under microaerophilic conditions and at a *p*H 7·4.

Transmission. Relapsing fever is transmitted by the agency of blood-sucking insects, particularly ticks and lice. Infection may be introduced directly into the tissues by the bite, but generally it follows contamination of wounds, caused by scratching or biting, with the excreta of the insect. The tick, once infected, may remain so throughout life, and the infection is transmitted hereditarily through several generations. Monkeys are susceptible to infection, and a condition similar to that seen in humans may be produced by intraperitoneal, subcutaneous or intradermal inoculation of the organisms. Rats and mice are more resistant, while guinea-pigs and rabbits are quite refractory.

The spirochætes are present in the blood in large numbers during the febrile stages, but as these subside the organisms become increasingly difficult to demonstrate. During the quiescent period they apparently lie dormant in the tissues, but they reappear in the blood during the relapses. The blood can be examined either as a stained film or by dark-ground illumination, when the actively motile spirochætes, in some cases straight and in others bent and resembling the letter S or C, are seen. For stained preparations Giemsa's method is satisfactory—the spirochætes are stained blue, but in some cases the staining is irregular.

Immunity. That the immunity following an attack of relapsing fever is only of a low order of efficiency is indicated by the fact that second attacks with the same treponema are not infrequent after 1–2 years. Antibodies—agglutinins, complement-fixing bodies and lysins—have, however, been demonstrated in the serum of recovered individuals. These antibodies are specific for the different treponemata responsible for the various types of relapsing fever; there is no marked cross-immunity.

The development of antibodies during the attack is considered to be responsible for the termination of the febrile periods. As antibodies are produced in sufficient quantities the spirochætes are rapidly agglutinated, disintegrated and ingested by leucocytes. Within a short time they disappear from the blood-stream and the temperature falls by crisis. Experimental work indicates that during the quiescent period the spirochætes are present in such tissues as the brain, where, owing to the relatively feeble blood supply, there is a low concentration of antibodies. At the end of the apyrexial period the antibodies in the blood have decreased and the spirochætes re-infect the blood and a similar cycle of events follows. The number of spirochætes in the blood tends to decrease with each successive relapse until the immunity produced is sufficient to prevent further invasion. It is, however, uncertain whether a true spontaneous cure actually takes place.

Prophylaxis. Measures adopted to prevent relapsing fever are attention to the general sanitation of the environment, personal cleanliness, and the avoidance of bites by ticks, lice, etc.

Treatment. Relapsing fever responds very well to the administration of arsenical compounds. A suitable dose may cure the disease. Some strains of the relapsing fever spirochætes have become arsenic-resistant, and for such infections potassium bismuth tartrate is recommended.

Vincent's Angina

Vincent's angina is an inflammatory condition of the mouth and throat, in which extensive ulceration and necrosis of the mucous membrane are generally found. The ætiology is uncertain, but an organism described by Vincent (1896), and now known as *Trep. vincenti*, is usually demonstrated, often in considerable numbers, in the lesions, together with a Gram-positive fusiform bacillus. *Trep. vincenti* is a thin organism (about 5–10μ in length) containing some three to eight irregular spirals; it is actively motile. It is Gram-negative, but does not stain very deeply or evenly by this method. It can be cultivated anaerobically at 37° C. on serum-agar, forming small, tenacious colonies. The pathogenicity of these two organisms is doubtful, and their *rôle* in the ætiology of Vincent's angina has not been definitely established. Similar organisms may be present in the mouths of normal individuals but never in the great numbers which are found in cases of Vincent's angina. In the tonsillar lesions Gram negative spirochætes and fusiform bacilli may be the only organisms seen. In cases of acute ulcerative gingivitis, unless the swab is carefully collected, these organisms may be masked by the numerous organisms usually associated with the gum margins. Lesions are not produced on subcutaneous injection into guinea-pigs. It has been suggested that the spirochæte and the fusiform bacillus represent different phases in the life cycle of one organism, but this does not appear probable on the evidence available. They appear to be distinct organisms which thrive in symbiosis.

Treatment. Cases tend to clear up satisfactorily with local treatment in the form of mouth washes and application of chromic acid solutions, combined with intravenous arsenical injections. The local application of penicillin in the form of lozenges has given good results.

CHAPTER XXXII

FILTERABLE VIRUSES

MANY diseases of man, animals, insects and plants, some of which exhibit a marked degree of infectivity, are caused by agents which cannot be studied by the ordinary bacteriological methods. These disease-incitants have certain properties which distinguish them from the ordinary bacteria; they are not visible by ordinary microscopy, they do not grow under the usual conditions of cultivation and they tend to pass through the bacterial filters. They thus constitute a distinct group of disease-agents and they have been designated *filterable viruses*, or more simply *viruses*.

Viruses have long been associated with the problems of immunity. In fact, active immunization was first widely used in the prevention of human disease in the case of two virus infections, viz., small-pox by Jenner (1800) and rabies by Pasteur (1885) respectively. Although the practice was for many years purely empirical, Pasteur had indicated the probable nature of the rabies virus when he postulated that he was dealing with an organism which he was unable to see with the microscope then available. Further development in the knowledge of the viruses was uneventful until Iwanowski (1892), a botanist investigating the mosaic disease of the tobacco plant, discovered the filterable nature of the responsible agent. These observations were confirmed later by Beijerinck (1899), who considered that he was dealing with a " Contagium vivum fluidum ".

About the same time a virus was discovered to be responsible for a disease of animals, when Loeffler and Frosch (1898) found that foot and mouth disease could be reproduced by a bacteriologically sterile filtrate of the vesicular lymph from the specific lesions. These observations stimulated the interest of many bacteriologists, and viruses have since been incriminated in the ætiology of many human and animal diseases. Nevertheless, it is only in recent years that any definite advance has been made in our knowledge of the nature of these agents, and this is still incomplete. Many methods have been used in their study, but only those yielding useful information need be considered here.

Filtration. As the designation of these agents indicates, filtration has been extensively employed in their study. It was early recognized that an important criterion of the viruses was their ready passage through bacterial filters. This criterion still holds for general application, but certain reservations are necessary. The process of, and the factors involved in, filtration have already been discussed (p. 63). It is sufficient now to point out that filtration is not a simple mechanical action governed only by the relative sizes of the pores of the filter and the particles to be filtered. Many factors are concerned, and the most important are: the composition and electrical charge of the filter; the pH and electrical charge of the material undergoing filtration; the amount of extraneous material in the suspension; the duration of filtration; the pressure employed; the temperature at which it is carried out. Viruses may be adsorbed on to the filter or on to albuminous material in the suspension and so fail to pass through a filter, which ought to be traversed. Irregular results have not infrequently been due to some variation in the filtration technique. The value of filtration in the study of viruses is therefore limited. The process cannot be used to determine with any accuracy the size of the virus particles, but by the use of the various grades of filter a means of obtaining a rough estimate of their dimensions is available.

Many viruses readily pass through the usual bacterial filters, such as Berkefeld " N " and Seitz " EK " types; others, *e.g.*, the vaccinia and herpes viruses, only traverse with difficulty the coarser types, such as the Berkefeld " V ", when special precautions are taken. Some smaller bacteria and various spirochætes may pass through the coarser filters more readily than the larger viruses. It consequently follows that filtration alone is not an absolute criterion for the identification of a virus.

Ultra-filtration. More precise studies of the size of different viruses have recently been made by the use of ultra-filtration, which was first employed for the study of colloidal suspensions by Bechhold (1907). This process involves the use of collodion membranes, which are less influenced by outside factors than the bacterial filters. Much of the early application of these membranes to the study of the viruses was unreliable owing to the use of faulty membranes, but in recent years a successful technique has been introduced by Elford. Some of the main points of this are: the grading of acetic acid membranes in terms of their average pore diameters, constant area of filtering surface, use of constant pressure and membranes supported on a perforated plate.

Many viruses have been studied by this technique, and marked variation has been found in their individual sizes : *e.g.*, psittacosis virus—0·275μ* = 275mμ* ; vaccinia virus—0·125–0·175μ = 125–175mμ ; influenza virus—100mμ ; poliomyelitis virus—8–12mμ and the foot and mouth virus—8–12mμ. The extremely minute size of the smallest viruses is more easily appreciated when it is realized that the size of a molecule of oxyhæmoglobin is 3–5mμ, while the average size of the *Staphylococcus* is 1μ = 1,000mμ.

The particulate nature of viruses is also indicated by the results obtained from centrifugalization—viruses have been deposited from virus suspensions by prolonged high-speed centrifugalization. Various types of centrifuge are available for this purpose and estimations of the particle dimensions of several viruses have been made. These figures agree very closely with results obtained by ultra-filtration, ultra-violet light photography and electron microscopy.

Microscopy. The small dimensions of viruses indicate that they are beyond the range of microscopic vision. It has been found that the limit of resolution using dark-ground illumination and visible rays is approximately 0·2μ ; under the usual laboratory conditions, however, the figure is probably never less than 0·25μ. Smaller objects may, however, be rendered visible by using photography with ultra-violet light and a special optical system of quartz lenses. As the results of ultra-filtration have shown that the greatest dimension of most viruses is no more than 0·15–0·175μ, it follows that the use of ordinary microscopical methods is of little avail. For this reason the viruses have been referred to as "invisible" or "ultra-microscopic" viruses. These terms are, however, misleading, and are now seldom employed.

Microscopy has three main applications in the study of viruses : (1) to observe the nature of the lesions produced in the various diseases, (2) to detect the presence of inclusion bodies, and (3) to determine the presence of elementary bodies.

(1) The nature of the lesions produced by the viruses may indicate the identity of the causative agent. Some viruses exhibit a marked affinity or tropism for special tissues, *e.g.*, the affinity for nerve-cells or neurotropism of the viruses of rabies and

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* μ or 1 micron = 1/1,000 mm. or a 1/25,000 inch.
mμ or millimicron = 1/1,000μ.

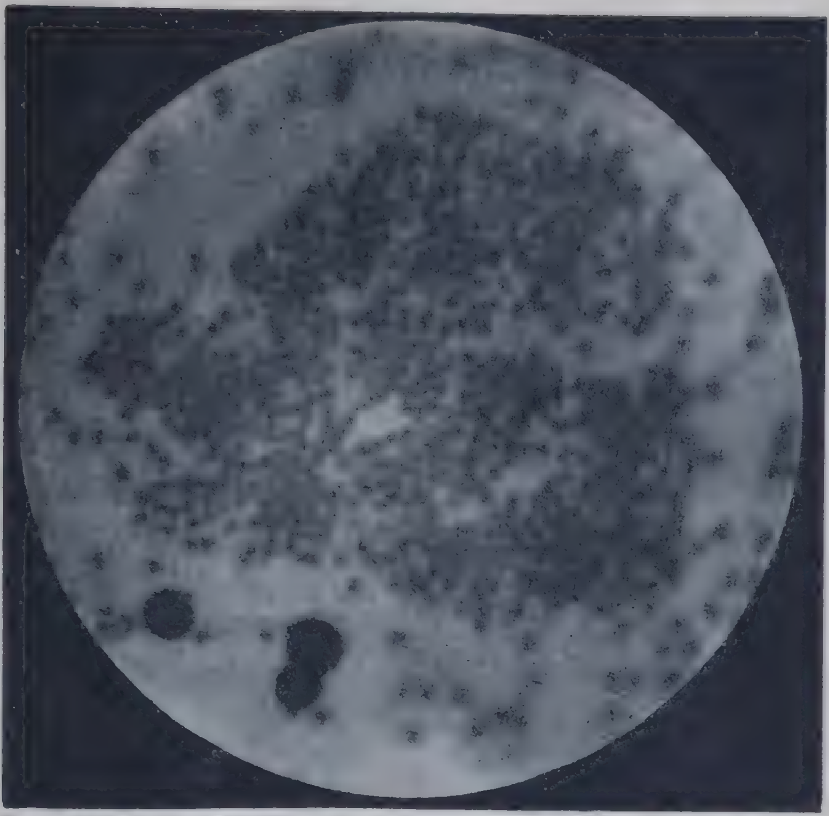
poliomyelitis. Also during the reaction between the virus and the tissue-cells characteristic changes are frequently produced. In some cases, *e.g.*, warts and molluscum contagiosum, there is a distinct tendency to cell proliferation ; in others, *e.g.*, small-pox and poliomyelitis, there is an inflammatory reaction with accompanying degeneration and destruction of the tissue-cells.

(2) The presence of abnormal bodies in the affected cells of many virus diseases has long been observed ; these bodies have been termed *inclusion bodies*. They may be present in either the cytoplasm or nucleus of the cells and are called respectively *cytoplasmic* or *intranuclear* inclusions. Inclusion bodies can frequently be demonstrated by ordinary staining methods, such as hæmatoxylin and eosin, but in a few cases a special staining technique is required. They vary considerably in size and shape. They are often of great diagnostic value, *e.g.*, the Negri bodies in rabies, but their nature and composition remain unsettled. Earlier views were that they were either of a protozoal nature or only simple reaction products produced by the cells. There is now much evidence to indicate that, in some cases at least, they are composed of large numbers of elementary bodies held together by a lipoidal matrix.

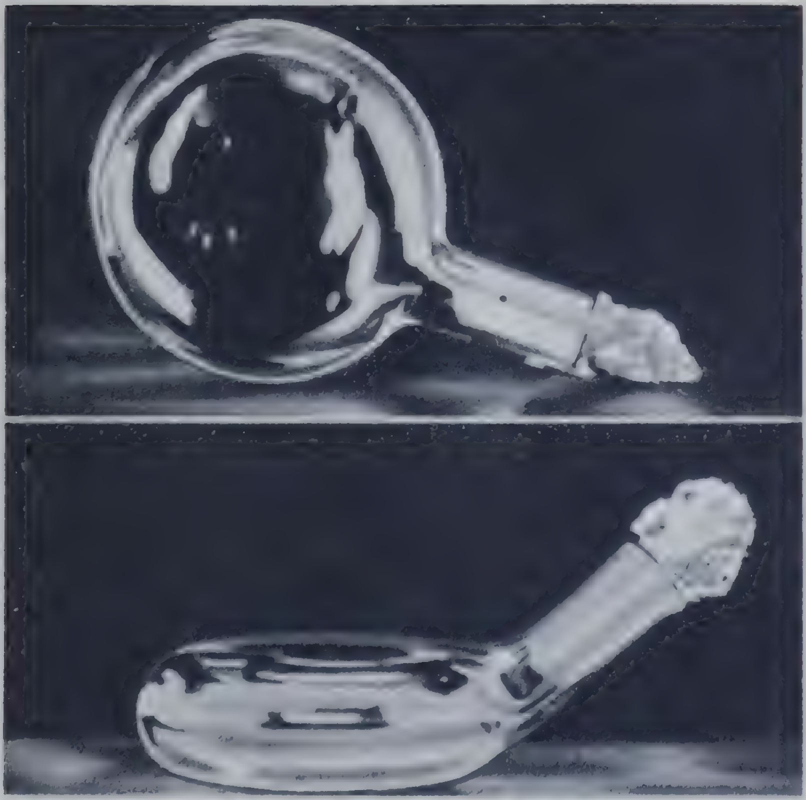
(3) In recent years attention has been re-directed to the presence of minute bodies in the exudates formed in certain virus diseases ; these had been observed by Borrel (1903) in fowl-pox, and by Paschen (1906) in vaccinial lesions, and were considered by these workers to be ætiologically related to the respective diseases. These *elementary bodies* have since been demonstrated in the lesions of many conditions, such as vaccinia, varicella, herpes zoster, fowl-pox and psittacosis. The bodies are agglutinated by the serum of animals recovered from the homologous disease. Pathogenicity tests also indicate that they are the ætiological agents of the various diseases. Elementary bodies are stained with difficulty ; it is necessary to use either the prolonged Giemsa's method or a special technique. When stained they come within the limits of microscopical resolution, probably through the deposition of the stain on the virus (Plate V.). Their relation to the inclusion bodies has already been mentioned.

The **electron microscope,** with which magnifications of 1–200,000 diameters can be obtained, is now being used for the study of viruses. Preliminary observations in U.S.A. indicate that viruses have a definite but heterogeneous structure, *e.g.*, the vaccinia virus is rectangular, resembling a brick, the influenza

PLATE V



ELEMENTARY BODIES.—The Borrel bodies of fowl-pox with three staphylococci; note that the diameter of the staphylococci, approximately 1 μ, is about 5 × that of the Borrel bodies (Giemsa). × 4,000.



Carrel Tissue Culture Flask, Type D (5 cm. diameter).

[*To face p.* 390.

virus is spherical, while the tobacco mosaic virus is rod-shaped, approximately 280 × 15$m\mu$.

Cultivation. The methods of cultivation used for obtaining growth of the bacteria have proved unsuitable for the multiplication of viruses. Claims of success have been made in certain cases, *e.g.*, the streptococcus of Rosenow and the so-called "globoid bodies" in poliomyelitis, but in no instance have these claims been substantiated. Several viruses have, however, been cultivated in the presence of living cells. At one time it was thought that actively growing cells were necessary; growth was then only obtained by means of tissue-cultures. The technique was simplified by the Maitlands (1929), who found that growth of the vaccinia virus took place in a medium composed of minced rabbit kidney and serum; no evidence of multiplication of the tissue-cells was obtained. This indicated that growth of the tissue-cells was not essential for virus multiplication. Many viruses have since been cultivated in this, or a similar medium, which is usually placed in special containers known as Carrel flasks (Plate V.).

A further advance has been the cultivation of viruses on the chorio-allantoic membrane of the developing chick-embryo (10–12 days old). The position of the membrane is determined by means of trans-illumination in a darkened room, and a flap is made in the shell over the membrane by means of a file or other suitable instrument. The flap is turned back, and the virus-suspension dropped on the membrane; the flap is then replaced and sealed with melted paraffin wax. After incubation at 37° C. for 2–3 days, the membrane is dissected out, ground up in saline and tested for the presence of virus by infectivity tests. This method is simple and has proved successful with the viruses of influenza, vaccinia, herpes, psittacosis, Rift Valley fever and many animal diseases. More recent developments have been the cultivation of viruses in the yolk-sac and amniotic sac of the chick-embryo.

Definite multiplication of viruses has thus only been obtained in the presence of living cells. Eagles and his colleagues have claimed success with certain cell-free media, but other workers have as yet been unable to confirm this work.

Cultivation *in vitro* has not been successful with all viruses. In a number of cases the only satisfactory method is the inoculation of susceptible animals. As some viruses only produce infection in man, their study is rendered extremely difficult.

Resistance. The viruses tend to behave as the vegetative bacteria in their resistance to destructive agents. As a rule they are readily destroyed by heat and oxidizing agents, but exhibit a marked resistance to drying, low temperatures, glycerine and weak concentrations of phenol (0·5 per cent.). In many cases viruses may be conserved in 50 per cent. glycerine at 0° C. for considerable periods. Under these conditions the poliomyelitis virus has survived over 8 years. With the doubtful exception of the lymphogranuloma venereum virus, the viruses are resistant to the action of the sulphonamides and penicillin.

Serology. Recovery from most virus diseases is accompanied by a high degree of immunity; second attacks are very uncommon. This resistance to re-infection is frequently associated with the presence of antibodies in the serum. These antibodies can also be produced by experimental infection or by immunization of animals with the modified virus. The precise nature and mode of action of the antibodies are unknown, but the available evidence indicates a resemblance to the bacterial antibodies. It has been suggested that they act on the tissue-cells rather than on the actual virus, but there appears to be little justification for this view.

The demonstration of antibodies is most satisfactorily carried out by means of the neutralization test. In this test a potent virus is mixed and left in contact with (*a*) the serum and (*b*) normal saline; the various mixtures are then injected into suitable animals. Those receiving the mixture (*b*) should exhibit the characteristic lesions, whereas those receiving mixture (*a*) remain unaffected if antibodies are present in the serum.

Complement-fixation and flocculation tests are carried out with some viruses, *e.g.*, those of vaccinia, herpes, influenza and psittacosis.

Viruses, in certain cases at least, appear to possess a complex antigenic structure. With suspensions of the vaccinia, psittacosis and influenza viruses, it has been found that the antigen involved in the complement-fixation test is a soluble substance and is distinct from that concerned in the neutralization test.

Pathogenicity. Filterable viruses are responsible for a number of important human and animal diseases. They appear to be essentially parasites; saprophytic members have never been described.

The incrimination of a virus in any disease-process is frequently a matter of considerable difficulty. The reproduction of the condition in an experimental animal and subsequent passage in series are essential. Failure to obtain passage of the infection indicates the action of a toxin rather than of a virus. The adaptation of a virus to a new host may be difficult, and during adaptation the virus may become greatly modified, *e.g.*, the street and fixed virus of rabies. It is also important, in adapting viruses to new hosts, to exclude the possibility of irregularities arising from the presence of spontaneous disease in the experimental animal.

Certain viruses produce disease only in man, experimental animals are quite refractory ; the participation of a virus in these conditions is concluded mainly by the elimination of other disease-agents. The presence of a virus cannot be definitely proved, although in the case of some viruses, *e.g.*, the virus of warts, tests have been carried out on human volunteers.

Classification. Our knowledge of viruses and the diseases produced by them is far from complete. Divergent views have been expressed as to the nature of viruses ; some workers consider that viruses approximate to the enzymes, *i.e.*, the dead, and others that they are essentially living bodies. In the case of at least the larger viruses the available data, particularly their particulate nature, morphology and power to multiply, suggest that in many respects there is a close relationship between the viruses and the bacteria. Many workers accept this relationship and consider that viruses are living, particulate bodies comparable with the bacteria, *i.e.*, viruses are small forms of bacteria.

It must, however, be noted that in the case of certain plant viruses, *e.g.*, tobacco mosaic virus, the virus activity appears to be closely related to, if not identical with, a protein of high molecular weight, a so-called " heavy " protein. The activity of the virus is retained in spite of considerable chemical and physical treatment and, moreover, the virus can be prepared in a crystalline form. In view of these observations this and related viruses are not considered as a living entity but as an autocatalytic substance, which alters the metabolism of susceptible cells so that they produce further supplies of the autocatalytic substance. The precise origin of the substance has not, however, been determined. While any human or animal viruses have not yet been conclusively identified with heavy proteins, these observations have opened a new field for virus research. It is thus impossible to state generally whether viruses are living or dead ; they appear to exhibit phenomena associated with both states.

Any final attempt at the classification of either the viruses or the virus diseases is quite impossible at the present time. Some viruses exhibit an affinity for certain tissues, and the terms " neurotropic ", " dermotropic " and " viscerotropic " viruses are frequently used in a loose descriptive sense to indicate various classes of these agents. It has recently been suggested that, in view of their large size, the viruses of lymphogranuloma venereum, psittacosis, and certain animal pneumonias form a distinct and separate group, which has been designated the " L.G.V.-psittacosis " Group.

A more satisfactory method is to classify the diseases according to our knowledge of the causative agent. The pathogenesis of some diseases has been worked out in detail by the examination of the condition at various stages in experimental animals, and the behaviour of the virus has consequently been studied in much detail. In other cases transmission experiments have been fruitless, and information about the responsible virus is incomplete. It has therefore been possible in some cases to obtain more informa-

TABLE XXX

Virus Diseases of Man

Accepted	Probable	Possible
Poliomyelitis. Rabies. Rabies encephalitis (Trinidad). St. Louis encephalitis. Yellow fever. Variola and alastrim. Vaccinia. Herpes. Psittacosis. Lymphogranuloma inguinale. Rift Valley fever. Epidemic influenza. Lymphocytic choriomeningitis.	Measles. German measles. Mumps. Dengue fever. Sand-fly fever. Epidemic encephalitis. Australian X disease. Herpes zoster. Varicella. Acute coryza. Infective hepatitis. Primary atypical pneumonia. Warts. Molluscum contagiosum.	Trachoma. Acute encephalomyelitis. Acute rheumatism. Hodgkin's disease. New growths.

tion about the causative agent than in other conditions. The following tentative grouping of the virus diseases is based mainly on this evidence and is only offered for convenience of discussion.

(1) Diseases of accepted virus origin.
(2) Diseases of probable virus origin.
(3) Diseases of possible virus origin.

There is no hard-and-fast line of demarcation between these groups, and much overlapping is doubtless present. The arrangement of the human virus diseases under this scheme is given in Table XXX.

A. ACCEPTED VIRUS DISEASES

Poliomyelitis

Acute anterior poliomyelitis or infantile paralysis is an acute infectious disease involving mainly the nerve-cells of the spinal cord and medulla. It was described in detail by Heine (1840) and Médin (1890), and is sometimes referred to as the Heine-Médin disease. The condition was first reproduced in the monkey by Landsteiner and Popper (1908).

The Virus. The ætiological agent of poliomyelitis is one of the smallest viruses ; by ultra-filtration methods its dimensions have been estimated at about 8–12$\mu\mu$. It readily passes through the bacterial filters and has not been cultivated *in vitro*. The claims of the globoid bodies and the streptococcus as ætiological agents of poliomyelitis have never been substantiated.

The only available experimental animal for general use is the monkey, in which the condition can be produced in order of reliability by intracerebral, intraneural, intrathecal and intranasal injection of virus-containing material. The experimental disease is comparable to the severe, fatal form in humans ; when the virus has become adapted to the monkey the mortality rate in these animals is approximately 95–100 per cent. Certain strains, *e.g.*, Lansing strain, can, after original adaptation to the monkey, be passed to the cotton-rat and then possibly to white mice.

In spite of the limitations of experimental work, the following properties of the virus have been established ; it is extremely resistant to freezing, drying, pure glycerine and 0·5 per cent. phenol ; it is, however, readily destroyed by heat over 45° C. and oxidizing agents. In nervous tissue the virus can be conserved for many years at 0° C. in 50 per cent. glycerine.

Distribution and Pathogenesis. Poliomyelitis is a widespread disease occurring in man only. It is chiefly encountered in children between the ages of 1 and 12 years, and may be present in either epidemic or endemic form. In some countries, *e.g.*, America, extensive outbreaks have been reported from time to time, but in this country sporadic cases or small outbreaks of very limited proportions only have been reported during recent years.

There is much epidemiological and experimental evidence to indicate that the disease is transmitted by droplet infection from cases or carriers, but some workers consider that the main route of transmission is the alimentary tract. The route taken by the virus after implantation in the naso-pharynx is not definitely known. Experimental work indicates that the virus enters the olfactory nerves by the exposed olfactory hairs and passes by the axones to the brain and then to the cord *viâ* the pyramidal tracts. During its progress the virus may attack any nerve-cells, but those usually involved are the anterior horn cells of the lumbar and cervical enlargements. In the experimental disease the initial lesions are found in the nerve-cells and not in the interstitial tissue, *i.e.*, the virus is essentially neurotropic. The lesions consist of degeneration, vacuolation and neuronophagia of the nerve-cells with a well-marked secondary perivascular and meningeal mononuclear infiltration. Before degeneration is well advanced intranuclear inclusions can be demonstrated in the affected nerve-cells.

The involvement of nerve-cells is indicated by the appearance of paresis and paralysis of the associated muscles. The mortality rate is variable, but tends to be about 10 per cent., and is due to damage to the respiratory and cardiac centres. When recovery occurs there is usually some associated permanent muscular impairment.

Immunity. Recovery from the disease is followed by a high degree of immunity ; second attacks are exceedingly rare. This resistance is often associated with the presence in the serum of antiviral bodies, which can be demonstrated without difficulty by means of the neutralization test. In regions where the disease is endemic, adults are seldom affected, and antibodies can be demonstrated in the serum of many adults who have displayed no previous manifestation of the disease. In contrast to this, it has been observed that in rural or isolated communities, where poliomyelitis is seldom seen, the introduction of the virus frequently gives rise to outbreaks involving individuals of all ages.

Moreover, antibodies are rarely present in the serum of the adult population. These facts indicate that in districts where the condition is endemic, sub-clinical infection is a common feature, and as a result the adult population becomes immunized. There is a well-recognized "abortive" form of the disease, in which muscular weakness is not found.

The theory of specific immunization by sub-clinical infection is not universally accepted. A few workers consider that the resistance of adults is due to a non-specific physiological maturation of the tissues. The evidence put forward to support this theory is, however, far from conclusive.

Prophylaxis. In recent years two methods of specific prophylaxis—active and passive—have been attempted. Passive immunization is carried out by the injection of convalescent human or immune horse serum, 5–15 c.c., intramuscularly; it is of short duration, and while good results have been claimed the figures are too scanty to enable an assessment of its value.

Methods of active immunization have been investigated extensively in America, and several vaccines have recently been recommended. In these the virus has been killed or attenuated by treatment with formalin, sodium ricinoleate or immune serum. As there is still some doubt concerning not only the safety of the vaccines, but also their capacity to induce immunity, further work is required before active prophylactic measures can be generally applied.

The chemical treatment of the olfactory mucosa in order to block the route by which the virus passes to the nervous system has been tried but not with any striking success. Intranasal spraying with zinc sulphate solutions (1 per cent.) has given the best results experimentally.

Therapy. The value of convalescent or immune horse serum as a therapeutic measure in poliomyelitis is difficult to analyse. Divergent results have been obtained by different workers. The main reasons for these irregularities are the difficulty of diagnosis in the preparalytic stages when the serum is most likely to be efficacious, the consequent difficulty in selecting suitable control cases for statistical surveys, and the not infrequent occurrence of complete spontaneous recovery of cases in which slight but definite muscular involvement has been observed.

Important non-specific therapeutic measures are rest in bed and spinal drainage.

Rabies

Rabies, or hydrophobia, is an inflammatory disease of the central nervous system transmitted to man by bites from infected animals, usually dogs.

The Virus. Although rabies is generally accepted as a virus disease there appears to be some doubt whether the ætiological agent is actually a virus. . Several workers consider that the responsible agent is a protozoon. Characteristic cytoplasmic inclusions, the Negri bodies, are present in the affected nerve-cells ; these are considered by some workers to represent a phase in the life cycle of a protozoon, *Glugea lyssæ*, and by others to be an agglomeration of a sporozoon, *Encephalitozoon rabiei*. In view of the uncertainty as to the nature of the suggested protozoon and the indefinite evidence advanced to support the respective theories, it appears advisable to consider the ætiological agent of rabies as a filterable virus.

The following properties of the infecting agent have been established : it is not readily filterable, as it only traverses a Berkefeld " V " filter with difficulty and has not been satisfactorily cultivated *in vitro ;* it resists glycerine and low temperatures, but its activity is decreased by drying, phenol (1 per cent.), oxidizing agents, ether and temperatures over 45° C.

Distribution and Infectivity. Rabies is primarily a disease of canine animals, from the bites of which man becomes infected. The virus is present in the central nervous system and the saliva of rabid animals. The incidence of the human disease is therefore dependent on the presence of infected animals. In Great Britain the disease has been eradicated by strict muzzling and quarantine laws, but in other countries, such as India, China and Russia, the disease is still prevalent.

The virus is introduced with the saliva into the subcutaneous tissues and is probably conveyed to the central nervous system by the regional nerve-axones. Rabies has been found to result in only some 35 per cent. of non-immunized individuals bitten by proved rabid animals ; once developed, however, the disease is invariably fatal.

The incubation period is long and subject to much variation (27–64 days) ; it depends largely on the proximity of the bite to the central nervous system. The main symptoms are irritation around the bite, fever, difficulty in swallowing and respiration, marked salivation and later delirium and paralysis.

The main lesions are found in the central nervous system. The nerve-cells show signs of degeneration and neuronophagia, while some, particularly those of the Hippocampus major, exhibit the characteristic cytoplasmic inclusions first described by Negri in 1903. Perivascular infiltration is also present.

Rabies can be reproduced experimentally in most laboratory animals, of which the rabbit is the one of choice. On the first intracerebral transfer of the street virus, *i.e.*, the virus obtained directly from the dog or human case, the incubation period is relatively long, but it is gradually decreased with successive transfers until it becomes constant at about 6 or 7 days. The virus has now become modified in that it exhibits a reduced infectivity for monkeys on subcutaneous inoculation and does not give rise to the formation of the Negri bodies. This modified virus is termed " virus fixe ", or fixed virus.

Immunity. Although the disease, once established, is always fatal, the development of symptoms can be prevented if artificial immunization is carried out during the early stages of the incubation period. This was one of the many important discoveries made by Pasteur. In view of the long incubation period of rabies after infection with the street virus, inoculation with graduated doses of the attenuated fixed virus is able to produce an immunity which aborts the original infection. Many modifications of the original Pasteur method, mainly in the mode of attenuating the fixed virus, have since been introduced. The inoculations are usually given subcutaneously on 14 or more successive days.

(1) **Pasteur's Method.** The spinal cord of rabbits inoculated with fixed virus is dried over KOH for varying periods of time, and when required saline suspensions are made of the cord. The most attenuated virus, *i.e.*, one dried for the longest time (14 days), is used for the first injections, this is followed by less attenuated virus until finally virus dried for only 5 days is used.

(2) **Hogye's Method.** Active virus diluted to 1/10,000 is used for the initial injections and the amount is gradually increased for the subsequent doses.

(3) **Babes' Method,** in which heated fixed virus is used ; at 80° C. for the first injection, then at 60° C., 45° C., and finally unheated.

(4) **Fermi and Semple's Method.** Virus inactivated by 1 per cent. phenol is used.

(5) **Remlinger's Method,** in which the virus is attenuated by treatment with ether.

The introduction of active immunization has decreased the incidence and mortality rate of rabies in a striking manner. The mortality rate has now been reduced to about 1 per cent. There is, however, no general agreement as to the most satisfactory type of vaccine. The results obtained by various workers indicate that the phenolized and etherized vaccines are probably the most valuable.

Acute Rabic Myelitis (Trinidad)

An outbreak of acute ascending myelitis occurred in Trinidad during the wet seasons of 1929–30 ; the mortality rate was 100 per cent. Hurst and Pawan found that the ætiological agent was the rabies virus, and considered that the original source of the infection was the cattle. A peculiar disease had been prevalent at that time among the cattle, and this was found to be most probably an infection with the rabies virus. There was no evidence of direct transmission of the infection from the cattle to man, but as many cases gave a history of bites by bats it appears probable that the vector was a vampire-bat.

Variola or Small-pox

Small-pox is a widely distributed eruptive disease which is frequently present in epidemic form.

The Virus. The virus of variola can be demonstrated in the vesicular fluid of the specific lesions by suitable staining methods. Its infectivity for experimental animals is low ; the most suitable animal being the monkey, in which, when injected subcutaneously, a well-marked local lesion and constitutional signs are produced. The inoculation of the rabbit merely results in a slight, local lesion with little, if any, general reaction. After several passages through the monkey the infectivity of the virus is increased considerably and well-marked lesions can be produced in the lower animals. As a result of adaptation to these animals the virus is definitely modified and is identical with the " vaccinia " virus ; *i.e.*, the vaccinia virus is the virus of variola modified by passage through the lower animals.

The vaccinia virus readily produces infection in the ordinary laboratory animals on cutaneous or intradermal inoculation, and it has consequently been the subject of extensive investigations. It is relatively large, 0·125–0·175μ, and therefore does not readily traverse bacterial filters ; it is resistant to glycerine, phenol, ether and low temperatures, but is readily destroyed by heat over 55° C. and potassium permanganate; it grows readily *in vitro* in the presence of living or surviving cells. In the vesicular fluid of the lesions small, coccal, *elementary* or *Paschen bodies* can be demonstrated, and these are considered to represent the virus.

Inclusions, termed *Guarnieri bodies*, are present in the cytoplasm of the epithelial cells of the lesions. On the intracerebral inoculation of a potent virus a meningo-encephalitis can be produced in rabbits and monkeys. The neurotropic strains are sometimes referred to as "neurovaccine". Following the inoculation of a potent virus by any route, it tends to generalize and lesions may be observed in various tissues.

Distribution and Infectivity. Small-pox was at one time one of the dreaded scourges. Large outbreaks with a high mortality rate were frequently reported; the lesions were severe, and on recovery pitting and scarring were the usual features. Following the introduction of prophylactic vaccination by Jenner there was a progressive decrease in the incidence and severity of the condition and at the present time the severe form is rarely encountered in this country.

The exact mode of transmission is not known, but most workers consider that the infection occurs through the agency of air-borne particles which settle in the upper respiratory tract. The virus is then carried by the blood-stream to the skin tissues, where it settles and multiplies in the capillary circulation.

The incubation period is about 9–15 days; the onset is characterized by fever, headache, vomiting and pains in the back. The rash appears on the third day in the form of discrete shotty papules mainly situated on the face, hands and legs; in 3 days the papules become vesicular, then pustular, finally drying up and after decrustation scars are left.

Alastrim. In recent years a mild form of variola has been observed in many countries. This disease is seldom fatal and has been termed *alastrim* or *para-vaccinia.* Some doubt was at first felt concerning the identity of this condition with the true variola. Experimental work has, however, shown that the viruses of these conditions are immunologically identical. Alastrim is consequently now accepted as an attenuated form of small-pox. Both conditions are related to cow- and horse-pox, which are produced by the vaccinia virus.

Immunity. An attack of small-pox or alastrim is followed by a high degree of immunity; second attacks are extremely rare.

A high degree of immunity can also be produced by artificial immunization. The immunity is associated with the presence of antibodies in the serum ; these can be demonstrated by neutralization, complement-fixation, agglutination and flocculation tests. The practice of active immunization by means of the vaccinia virus was introduced as a general measure mainly through the efforts of Jenner, who investigated the farm-workers' contention that an attack of horse- or cow-pox rendered them immune to small-pox. Vaccination, as the process is now known, is carried out at the present moment under State control ; it was first officially adopted in England in 1853. The living virus is employed, but the type employed in different countries varies ; in this country calf-lymph is used, on the Continent the potent neurovaccine is frequently employed, and in America virus cultured *in vitro* has recently been used ; the latter preparation has the great advantage in that it can be readily obtained in a sterile condition.

In England the preparation of calf-lymph is controlled by various Acts and Regulations. Attention is directed to the condition of the calves and also to the purity and potency of the final product. The vaccinia virus is seeded on the scarified abdomen of young healthy calves, 4–6 months old ; on the fifth day this area is scraped off and titurated with 50 per cent. glycerine and distilled water. The pulp is sealed and kept at − 11° C. ; cultures, aerobic and anaerobic, are made at intervals. The lymph is ready for issue only if the potency tests are satisfactory, if streptococci and anaerobes are absent, and if the aerobes are less than 5,000 per c.c.

Vaccination in man is usually carried out in infancy by cutaneous scarification ; the lymph is placed on the cleansed skin and light scarification made through it. A local vesicular lesion is produced in 5 or 6 days ; this passes through a pustular phase and finally dries up, leaving a scar. The immunity produced by this form of vaccination is sufficient to protect against the slight risk of infection in this country ; re-vaccination at intervals is desirable and should invariably be carried out in the event of an outbreak in the region. Service personnel, who are likely to visit countries where small-pox is endemic, are re-vaccinated at frequent intervals (3–5 years) ; in the presence of an outbreak re-vaccination with three insertions of lymph is recommended.

In immunized individuals, successful re-vaccination is followed by three types of reaction depending on the immunity state of the individual : (1) *primary reaction* which is the classical type and indicates lack of immunity, (2) *accelerated* or *vaccinoid reaction*, in which there is a mild form of reaction, complete in 8–10 days ; this indicates a moderate degree of immunity, and (3) *immediate reaction* in which a raised area of erythema appears, reaches a maximum within 72 hours and disappears rapidly ; this is indicative of a high degree of immunity.

Complications are unusual, but occasionally after primary vaccination of adults an encephalo-myelitis develops some 11–16 days after the vaccination. While there is little doubt that the nervous condition is definitely associated with the process of vaccination, it appears equally certain that the vaccinia virus is not the direct cause of the lesions in the nervous system (see p. 422). In order to reduce the risk of such complications, the Ministry of Health (1928) recommended *inter alia* that one insertion with the minimum of trauma should be given ; primary vaccination should be performed in infancy between the ages of 2–6 months ; and two medical inspections instead of one should be made subsequently.

Owing to the possible occurrence of these serious complications and the mild form of the recent type of small-pox, some authorities recommend that vaccination should no longer be enforced ; they consider that the natural infection is less hazardous than the immunization process. In consequence only some 40 per cent. of children in this country are now vaccinated in infancy. It is, however, possible for an un-immunized community to develop under such conditions, and the introduction of a virulent virus into such a population may have serious consequences. The latter event is rendered more probable by the increase and development of air transport, as it is now easier for individuals incubating the infection to gain admittance into the country. Several small outbreaks have recently occurred here as a result of the arrival from overseas of men either during the incubation period of the disease or exhibiting mild atypical clinical manifestations. It is therefore important that the vaccination of infants should be universally practised.

Herpes Simplex

Herpes simplex is an acute eruptive disease of the skin and mucous membranes, occurring frequently on the face and genitalia.

The disease is very mild ; a crop of vesicles appears after local irritation, the serous fluid exudes and forms a crust, which falls off, leaving no scar. Herpes frequently recurs in certain individuals who appear to harbour the virus in the naso-pharynx; it is activated on stimulation from varied sources.

The Virus. Herpes can be reproduced experimentally in most laboratory animals by corneal, cutaneous or intradermal injection of the virus. The following properties of the virus have been determined: it is resistant to 50 per cent. glycerine and low temperatures, but is readily destroyed by heat over 50° C. It is relatively large, $0{\cdot}1$–$0{\cdot}15\mu$, and does not readily pass through bacterial filters; it can be cultivated *in vitro* in the presence of living cells.

With some strains a meningo-encephalitis has been produced in rabbits; this suggested to some workers that two distinct strains of the virus existed, one being dermotropic and the other neurotropic. The general opinion is, however, that the affinity of the virus for different tissues is merely a question of virulence and does not indicate the presence of two distinct types of the virus. The so-called "neurotropic" strains have been shown to be more virulent than the "dermotropic" strains. The relationship of the herpes virus to epidemic encephalitis is discussed later (p. 417).

Immunity. The development of immunity in man is uncertain. A second crop of vesicles may occur within a short interval of the first crop. Antibodies have been demonstrated in the serum of many individuals, and the presence of these bodies appears to exercise little influence on the development of the vesicles. The action of the virus is possibly too superficial to be affected by them. A relatively high degree of immunity can, however, be produced in rabbits by active immunization. The virus of herpes simplex is immunologically distinct from that of herpes zoster.

Yellow Fever

Yellow fever is an acute febrile disease occurring usually in tropical regions in epidemic and endemic forms.

The Virus. The failure of workers to obtain suitable experimental animals considerably handicapped investigations, but by the use of human volunteers the American Yellow Fever Commission (1900) found that the infecting agent was filterable and was conveyed by mosquitoes. Many years later Stokes, Bauer and Hudson (1928) reproduced the disease in Asiatic monkeys and more detailed examinations of the condition became possible. The size of the virus has been estimated to lie between 0·017–0·028μ; it passes through the Berkefeld "V" and "N" filters, but not the "W" type. The virus is resistant to the action of 50 per cent. glycerine, drying and low temperatures, but is readily destroyed by heat over 55° C. and formalin. Theiler (1930) demonstrated that the virus possessed neurotropic properties, as he produced and passed serially an encephalitis in white mice by intracerebral inoculation. An encephalitis has since been produced in a number of rodents.

Noguchi (1919) isolated a spirochæte, *Lept. icteroides*, from a disease thought to be yellow fever. This organism has since been found to be identical with *Lept. icterohæmorrhagiæ*, and consequently the disease investigated by Noguchi was most probably Weil's disease and not yellow fever.

Distribution and Infectivity. Yellow fever is widespread in tropical and sub-tropical countries, being particularly common in South America and West Africa. The disease is usually transmitted from man to man by the mosquito, *Ædes ægypti*, or allied species. Extensive anti-mosquito campaigns in South America practically eliminated the disease but, when complete eradication appeared likely, a fresh outbreak occurred in an entirely fresh region and the origin of this could not be determined. Extensive investigations followed in which two important diagnostic measures were introduced: viz., the mouse-protection test and viscerotomy, in which pieces of liver are removed from cadavers by means of the viscerotome and subjected to histological examination. As a result of this investigation it was established that there is a special type of yellow fever which is primarily a disease of jungle-animals, probably monkeys, and which is not transmitted by *Ædes ægypti*. This type can not be distinguished clinically, pathologically or immunologically from the usual type of the disease; its mode of spread has not yet been elucidated.

Laboratory infection has been reported following the handling of infected material; the virus either penetrates the unbroken skin or enters through minute abrasions.

White people are particularly susceptible, and the disease usually runs a typical course. After an incubation period of 3–6 days there is a sudden onset with fever, headache and muscular pains, which last about 3 days; then the temperature falls to about 98° F., while jaundice, albuminuria and hæmorrhages into the intestinal tract are observed. The mortality rate varies from 20–90 per cent.; death may occur in the early stages, but it is more common on the fifth or sixth day.

On autopsy, jaundice, hæmorrhages into the skin and mucous membranes with fatty and necrotic changes in the liver and kidney are found; nuclear inclusions are present in the liver-cells.

The virus is present in the blood during the late incubation period and the first 3 or 4 days of fever. Mosquitoes only become infected when they suck the blood during these stages, but they do not usually become infective for man for at least 12 days; the time varies with the atmospheric temperature. Once infected the mosquito remains so indefinitely.

Immunity. An attack of yellow fever is followed by a high degree of immunity, second attacks being rare; this state is associated with the presence of antibodies in the serum which can be demonstrated by the neutralization or protection test in mice. Neutralization tests have been used extensively in epidemiological surveys of yellow fever and results indicate that the condition is widespread in Africa and South America. It is well recognized that the inhabitants of regions where the condition is endemic are resistant to infection. Recent studies have shown that the sera of these natives contain the antiviral bodies; this suggests that they undergo mild sub-clinical infections during childhood. Antibodies have also been demonstrated in the serum of monkeys in regions where the condition is endemic, and this suggests that monkeys may act as alternative hosts for the virus.

Prophylaxis. Important preventive measures are the suppression of mosquitoes and their exclusion from districts which are free from the disease. The increasing use of air transport is a possible danger in tropical regions as a means of introducing not only infected mosquitoes, but also infected humans incubating the disease into countries at present free from yellow fever.

Active immunization is now essential for individuals visiting certain tropical regions such as W. Africa; various vaccines have been tried: one widely used, but now given up, consisted of the living neurotropic mouse virus and immune serum. This type of vaccine tended in a number of instances to give rise to severe reactions, such as pyrexia, headache and muscular pains, and was responsible for many cases of hepatitis, the virus of which was considered to be present in the serum constituent of some batches of vaccine. This type of vaccine has now been replaced by an attenuated pantropic strain grown in a chick-embryo culture medium and the results have been highly satisfactory. The reactions are much less severe while the degree of immunity induced appears to be at least as high as that given by the neurotropic virus and immune serum vaccine; it is considered to be effective for four years.

Psittacosis

Psittacosis is primarily an infectious disease of birds, particularly parrots and budgerigars, which is transmissible to man, giving rise to severe pulmonary symptoms.

The Virus. Nocard (1892) isolated a member of the Salmonella group, *Bact. aertrycke*, from cases of psittacosis, and this organism was generally accepted as the ætiological agent of the disease until Bedson, Western and Simpson (1930) found that the responsible agent was a filterable virus. These workers reproduced the disease in budgerigars by injecting material obtained from infected humans and parrots. These observations were confirmed, and experimental infection was later produced in a number of animals, including mice, guinea-pigs and monkeys. The virus is relatively large (200–300$m\mu$) and only traverses the coarse bacterial filters with difficulty; it is resistant to glycerine and low temperatures. Elementary bodies are found in the specific lesions and also in tissue-cultures; various developmental stages have been reported.

Distribution and Infectivity. The association of human psittacosis with parrots has long been recognized. The disease was practically unknown in this country until a number of cases were reported in 1929–30, at which time cases appeared in many other countries. The source of the infection appears to have been South America, where about that time there was an extensive outbreak of the condition among the parrots. The majority of the human cases gave a history of close contact with sick birds, infection probably occurring by inhalation. The virus apparently possesses a high degree of infectivity for man as, although transmission of infection from man to man is not common, many instances of laboratory infection have been recorded.

The symptomatology is rather indefinite; a pneumonia of the influenzal type frequently develops. The mortality rate in different outbreaks has averaged about 20 per cent. On autopsy the pathological findings are rather vague; changes, such as consolidation, are seen in the lungs.

Infection can be produced experimentally in mice, parrots, budgerigars and monkeys.

Immunity. An attack of psittacosis is usually followed by a high degree of immunity; second attacks in man have not been reported, while recovered parrots are resistant to re-infection. Antibodies have been demonstrated in the serum of recovered cases by means of the complement-fixation test. Active immunization of mice has been carried out by the use of the formalized virus.

Rift Valley Fever

A disease causing a heavy mortality in newly born lambs appeared in Rift Valley, Kenya Colony, during 1930. On investigation the ætiological agent was found to be a filterable virus which was demonstrated in the blood, liver and spleen of infected animals by the intravenous inoculation of the tissue suspensions into healthy sheep. Man appears to be highly susceptible, as during the outbreak the majority of natives engaged in herding the sheep experienced a febrile illness with joint pains. Many laboratory workers have experienced similar attacks following the examination of infected material. A high degree of immunity appears to follow an attack.

St. Louis Encephalitis

An outbreak of encephalitis occurred in St. Louis and Kansas City in 1933 and affected more than 1,000 people. The mortality rate was about 20 per cent., being relatively high in individuals over the age of 40 years. This condition was considered to be a form of epidemic encephalitis, but later a virus was isolated from the brain-tissues of fatal cases. This virus produced an encephalitis in mice and monkeys on intracerebral inoculation; its relationship to the other neurotropic viruses has not yet been determined. It is considered to be closely related to the virus obtained from cases of encephalitis reported in Japan during 1924 and designated the type B encephalitis virus. Serological differences have, however, been demonstrated in the two viruses.

Equine Encephalomyelitis

Small outbreaks of encephalitis have recently been reported in U.S.A.; in one of these 46 cases were observed with 33 deaths, approximately half the cases occurred in children under 5 years of age. A virus was isolated from the brains of fatal cases by the inoculation of mice. This virus was later found to be identical with the Eastern type of equine encephalomyelitis virus. From other outbreaks the Western type has been isolated. The onset of the disease was sudden with pyrexia, irritability, neck rigidity, convulsions; most patients became semi-comatose while fatal cases developed deep coma. Pathological changes were found in the brain and cord, consisting of nerve cell destruction with perivascular and meningeal infiltration with polymorphonuclear and large mononuclear cells.

Epidemic Influenza

Influenza is an acute febrile disease which may appear in pandemic, epidemic or sporadic form. It is characterized by its sudden onset, marked prostration of the patient and the tendency to complications, such as bronchopneumonia. It has a worldwide distribution. The pandemic of 1918–19 is considered to have involved some 500,000,000 people and to have been responsible for at least 15,000,000 deaths.

Although influenza is such a common condition and has been subjected to extensive investigations in most countries, the ætiology of the epidemic form has only recently been determined. Many theories have been propounded, but it is only necessary to consider here the two most important agents, viz., (1) *H. influenzæ*, and (2) a virus.

(1) *H. influenzæ*, or the influenza bacillus, was first isolated and described as the ætiological agent of influenza by Pfeiffer in 1892. As it was a new species with certain characteristic features its relationship to the disease was then accepted. Much of the subsequent work failed to confirm the original claims of Pfeiffer, and at the present moment there is much evidence to indicate that the influenza bacillus plays only a secondary *rôle* in influenza. The organism has not been isolated from all cases of the disease, it is more readily obtained from complicated than from uncomplicated influenza, it is present in the throats of many normal individuals, and it has not reproduced with any regularity a typical attack of influenza on inoculation into human volunteers or experimental animals. There is, however, no doubt about its pathogenicity for man, as it is frequently the causative agent of post-influenzal bronchopneumonia (see Chapter XXII.).

(2) **Virus.** The failure to incriminate the influenza bacillus

suggested that a virus might be the ætiological agent of influenza. The earlier work was rather tentative and inconclusive, but, in 1933, Smith, Andrewes and Laidlaw obtained definite evidence of this relationship when they were able to transmit in series to ferrets a febrile disease by the nasal instillation of throat washings from influenzal patients. It was later found that the intranasal instillation of the ferret virus into mice under ether anæsthesia produced a bronchopneumonia which usually ended fatally in 3–5 days. Direct passage of the virus from human to mouse tissue was not, however, possible.

These observations were soon confirmed by workers in various parts of the world, but the influenza virus was only isolated from epidemic forms of the disease. As in some instances attempts to isolate the virus from obvious outbreaks of epidemic influenza were unsuccessful, it was thought that there might be several distinct types of the influenza virus. It was therefore decided to designate the virus already isolated as the influenza A virus. Confirmation of this view was obtained in 1940, when Francis and his colleagues isolated, from an epidemic in California, a virus which was antigenically distinct from the A virus. This was termed the influenza B virus and it has since been responsible for outbreaks in many countries, including the one recently experienced in this country.

Attemps to infect ferrets with material from sporadic cases have been unsuccessful. This is not altogether surprising, as in many cases of so-called "sporadic influenza" the diagnosis is made without much justification. The term is merely a convenient expression for all vague and atypical infections of the upper respiratory tract.

The influenza virus is relatively large, being about $0{\cdot}1\mu$ in its largest diameter. It grows readily in tissue-culture or on the chorio-allantoic membrane of chick embryos. Antibodies can be readily demonstrated by either the neutralization test or complement-fixation test in the serum not only of convalescents but also of many normal adults. In the neutralization test the elementary bodies, *i.e.*, the virus bodies, are involved; while in the complement-fixation test the antigen is a soluble substance present in virus suspension and apparently produced by the virus during multiplication. A further method is the *erythrocyte agglutination test*, which was described by Hirst (1941) and is now extensively used: the influenza virus is able to agglutinate the red blood corpuscles of the chick through adsorption of the virus on to the cells ; this action is neutralized by serum containing the homologous antibodies.

By serological tests it has been found that there is a close antigenic relationship between influenza A viruses isolated in England, America, Australia and on the Continent. By means of the neutralization test it has also been found that strains of this virus exhibit minor antigenic differences, which are frequently difficult to detect ; a method of sub-classification is not yet possible on these lines. The swine virus which, with *H. influenzæ suis*, is responsible for swine influenza, can be readily distinguished from human strains by neutralization tests.

Work on specific prophylaxis and therapy is still in the preliminary stages and a final opinion is not possible. From results so far obtained it appears that convalescent serum or immune horse serum is not of great value either as a prophylactic or therapeutic agent. Mice can be immunized with the virus killed by heat at 57° C. or by formalin. The results of active immunization in man are limited in number. The injection of the vaccines undoubtedly stimulates antibody production and recent trials in America indicate that they also afford some protection against epidemic influenza.

Lymphogranuloma Venereum : Climatic Bubo

During the past few years attention has been directed to the ætiology of lymphogranuloma venereum (lymphogranuloma inguinale). This is a veneral disease, which is characterized by adenitis of the inguinal glands and the presence of a small primary lesion on the external genitalia. Although rare in this country it is common on the Continent and in tropical regions. Material obtained from infected glands and found to be bacteriologically sterile gives rise to a meningo-encephalitis on intracerebral inoculation into monkeys and mice. Findlay has carried out detailed investigations and considers that climatic bubo and lymphogranuloma venereum are identical conditions and are both caused by a filterable virus.

A useful diagnostic measure is the *Frei Test*. This test is carried out by the intradermal injection of a saline extract of either infected lymph nodes or the brains of infected mice, heated at 60° C. In a positive reaction there is local erythema, with or without induration or vesicle formation, after 24 hours and persisting for 5 to 10 days. A preparation containing virus grown in culture and termed " *lygranum* " is now available for this test.

Acute Lymphocytic Chorio-meningitis

In recent years a virus has been isolated in America and this country from certain cases of so-called aseptic meningitis. This condition is normally non-fatal and may run a long course, perhaps several months; complications are unusual. The disease can be transmitted to mice, guinea-pigs and monkeys by intracerebral inoculation of the cerebrospinal fluid collected from cases of the condition. A leptomeningitis develops after an incubation period of 5–12 days.

The virus is relatively large, 200–250$m\mu$, and consequently does not filter readily, but it can be deposited by high-speed centrifugation. The virus can be grown by cultivation on egg-membranes. Antibodies, demonstrated by neutralization tests, are present in the serum of humans or animals recovering or recovered from the infection. Irregular results may, however, be obtained from these tests as several antigenically distinct strains of the virus have been isolated.

The virus has also been isolated from spontaneous disease in mice and consequently experiments in which mice are concerned must be strictly controlled.

B. PROBABLE VIRUS DISEASES

Measles

Measles is an acute febrile disease of extreme infectivity occurring generally in children in epidemic form. The infection is probably transmitted mainly by droplet infection. The condition tends to take the following course: after an incubation period of about 14 days, the onset is characterized by fever, sore-throat and coryza, with the appearance of white spots, termed "Koplik spots", on the buccal mucous membrane. A papular rash appears on the fourth day, first around the neck and spreading over the body; the tongue is thickly coated. Three or four days later the symptoms subside and desquamation commences. The mortality rate depends largely on the incidence of complications, the most frequent of which are bronchopneumonia, otitis, meningitis, nephritis and endocarditis.

Ætiology. The identification of the ætiological agent of measles has been rendered very difficult by the failure to obtain an experimental animal in which the typical disease can be reproduced. Monkeys have been used by many workers with varying degrees of success; other animals appear to be unsuitable. It is therefore not surprising that various agents have been incriminated in the ætiology of the disease. Blake and Trask (1921) reproduced a measles-like condition in monkeys by inoculating into the upper respiratory tract of the animals human naso-pharyngeal washings and filtrates collected in the early stages of the disease. The condition also developed in susceptible humans and monkeys following the intravenous injection of sterile human blood collected from early cases of measles. These observations suggest the participation of a filterable virus. Other workers have, however, isolated and incriminated certain bacteria : Caronia obtained a minute Gram-negative diplococcus ; Tunnicliff, Ferry and Fisher and others have isolated green-producing streptococci. These claims have not been confirmed by the majority of workers, who consider that, in some cases, these organisms are merely members of the normal flora of the human upper respiratory tract. The cultivation in the developing chick-embryo of a virus from the blood and naso-pharyngeal washings of cases has recently been claimed by workers in America.

Immunity. Measles is followed by a high degree of immunity ; second attacks are extremely rare. In urban communities most adults are immune, and as a number usually give no history of a previous attack of measles, this suggests that sub-clinical infection is a common feature in such communities. The introduction of measles into a virgin population generally results in a widespread outbreak involving individuals of all ages.

Serum in Prophylaxis and Therapy. The presence of antibodies in convalescent or adult serum is difficult to demonstrate, but it is almost certain that they are present, as the use of convalescent serum has proved satisfactory both as a prophylactic and therapeutic measure.

Antisera have also been prepared in horses by the inoculation of the various organisms claimed as ætiological agents of the disease. Their clinical application does not, however, appear to be particularly successful.

The value of convalescent and adult serum was investigated in detail by the London County Council (1933), and these sera are now being widely applied. They can be used to produce either (1) complete protection or (2) attenuation of the disease in individuals exposed to infection.

(1) Protection is advised for young or debilitated children ; the serum is given intramuscularly within 4 days of exposure to infection ; the dose recommended for children under 3 years of age

is 10 c.c., and for those over 3 years it is 20 c.c. This immunity is passive, and consequently only lasts a short time, some 3–4 weeks.

(2) Attenuation of the infection is of greater practical value. It is followed by the development of an active immunity, as the infection is not arrested but only modified by the administration of the serum; the incubation period is prolonged, the fever is mild and the rash slight. The serum in the above doses is given either after the fourth day and before the ninth day following exposure to infection. A similar result may be obtained by giving half the dose before the fourth day. If the administration of serum is delayed beyond 9 days after exposure to infection the course of the disease and the incidence of complications are not influenced.

Gamma globulins are now being used for this purpose; excellent results are reported from America.

German Measles

German measles is a mild infectious disease characterized by the appearance of a shotty papular rash on the face and body with a mild degree of pyrexia and enlargement of the cervical glands.

Owing to its mild nature, German measles has not been subjected to much extensive investigation and its ætiology is unknown. One attack is followed by a high degree of immunity.

Although there is a clinical resemblance to measles, the two conditions are immunologically distinct and therefore appear to be ætiologically separate.

Mumps

Mumps is an acute febrile condition characteristically involving the parotid glands and occurring most frequently in childhood. Mumps is transmitted mainly by droplet infection. The incubation period is long (14–21 days) and the initial symptoms are pyrexia and headache with swelling and tenderness of one or both parotid glands. The condition usually subsides in a few days, but such complications as orchitis, pneumonia, endocarditis and local suppuration may supervene.

There is now much evidence to incriminate a virus as the ætiological agent of mumps. Earlier work was suggestive, and Wollstein (1916), by the intratesticular and intraparotid inoculation of cats with the filtered saliva from cases of mumps, produced local swelling and tenderness with pyrexia ; this condition was passed serially through cats. Johnson and Goodpasture (1934) injected filtered saliva from cases of mumps into the parotid glands of monkeys and reproduced a condition comparable to mumps in man. They later found that the virus responsible for the monkey condition was identical with that causing human mumps.

Other agents have also been suggested, *e.g.*, Kermogant (1925) obtained results which indicated to him that a spirochæte was responsible. Other workers have failed to substantiate his claims.

An attack of mumps usually confers a high degree of immunity, but second attacks have occasionally been reported.

Dengue Fever

Dengue fever is a mild mosquito-borne disease occurring in the tropics. Alarming epidemics have been reported, but in view of the mildness of the symptoms the mortality rate is very low. The condition is transmitted from man to man by the agency of mosquitoes, principally *Ædes œgypti*.

Man only appears to be susceptible, attempts to reproduce the disease in experimental animals have been unsuccessful. The disease has been transmitted to man by means of infected mosquitoes and also by the inoculation of blood collected during the febrile stages of the condition. It has also been shown that the ætiological agent passes through Berkefeld filters. Attempts have been made to associate dengue fever with yellow fever, but these have proved unsuccessful.

Other agents to be incriminated are globoid bodies and the Rickettsiæ, but the relationship of these agents to the disease has never been determined with any certainty.

The degree of immunity following an attack of dengue fever is uncertain. It is usually considered to be of a low order and of short duration ; recent work suggests that this is not the case, but that a high grade of immunity results from an attack.

Sand-fly or Pappataci Fever

Sand-fly fever is a tropical disease transmitted by sand-flies and characterized by its sudden onset and short duration. Recovery is as a rule rapid and complete, the disease is never fatal.

Laboratory animals are not susceptible to the disease. Experiments on human volunteers suggest that the ætiological agent is a virus which is present in the blood during the febrile period.

An attack is followed by a high degree of immunity.

Epidemic Encephalitis or Encephalitis Lethargica

Epidemic encephalitis is an infectious disease of the central nervous system involving mainly the grey matter of the brain. It was first recognized as a clinical entity in 1917 by v. Economo, who described a small outbreak in Vienna ; lethargy was a prominent feature, and he termed the disease *encephalitis lethargica.* Subsequently outbreaks of varying severity were reported in most parts of the world ; in recent years the incidence has decreased considerably and the condition is now rarely seen. The peak year in this country was 1924, when 5,036 definite cases were reported.

Epidemic encephalitis displays a wide range of symptoms. This is not surprising, as lesions may be found in any part of the upper central nervous system. Many excellent clinical accounts are available for those desiring a detailed description of the disease.

The onset may be sudden or gradual. Prominent symptoms are pyrexia, headache, lethargy during the day, nocturnal insomnia, visual disturbances such as diplopia and blurred vision ; there may also be delirium, convulsions, myoclonus, choreiform movements and hiccup. The mortality rate is about 30–40 per cent., and serious sequelæ result in a similar number of cases.

On autopsy the chief lesions are generally found in the grey matter of the mid-brain, pons and medulla, and consist of perivascular and diffuse infiltration with degeneration of the nerve-cells and occasional neuronophagia.

Ætiology. In spite of much investigation by workers in different countries, the nature of the ætiological agent remains unknown. The chief reason for this is that a satisfactory experimental animal has not been found. The following agents have been incriminated :

(1) **Distinct Virus.** Several workers claimed to have reproduced the disease in rabbits and that the ætiological agent was a virus. Later work has shown that in some cases the agent responsible for the experimental disease was the herpes virus and in others a protozoon, *Encephalitizoon cuniculi*, which causes

spontaneous encephalitis in rabbits. In the majority of cases when the intervention of the other agents has been definitely excluded the results have been negative.

(2) **Herpes Virus.** The herpes virus has been isolated on a few occasions by various investigators from the brain tissue and naso-pharyngeal washings collected from cases of encephalitis. Some workers have consequently concluded that the herpes virus is ætiologically related to the condition. In support of this theory they showed that the so-called "neurotropic" strains of the herpes virus could produce an encephalitis in rabbits.

Other workers have been unable to confirm these results. Moreover, Flexner (1923) isolated from the cerebrospinal fluid of a case of neurosyphilis a strain of herpes virus which was able to produce an encephalitis in rabbits. Flexner and his colleagues were also unable to demonstrate any difference, other than in virulence, between the so-called "neurotropic" and "dermotropic" strains of the herpes virus. They consequently were unable to accept the view that the herpes virus was the ætiological agent of epidemic encephalitis. Other facts, which do not support this relationship, are: (*a*) the characteristic inclusions of herpes have not been demonstrated in lesions of human encephalitis; (*b*) the herpes virus has been isolated from the naso-pharyngeal washings of normal individuals; (*c*) facial herpes has not been observed with any frequency in cases of encephalitis.

The case for the herpes virus is thus far from conclusive; claims have recently been made that on intracerebral inoculation of Cebus monkeys an encephalitis resembling the human form was reproduced. These observations require further investigation.

(3) **Other agents,** such as the influenza bacillus and various streptococci, have also been suggested, but there appears to be little justification for these claims.

The position of the ætiology of the disease has been suitably expressed by the Matheson Commission (1932): "Again it is necessary to conclude that the question of the ætiology of epidemic encephalitis is still unanswered". The general opinion is that a distinct virus of low pathogenicity is responsible; this has been designated the type A encephalitis virus.

Australian X Disease

A peculiar acute disease of the central nervous system appearing in Australia during 1917 and 1918; it later reappeared in 1925

The disease appears to have been strictly limited to Australia. There was an abrupt onset with pyrexia, headaches passing rapidly to convulsions, delirium and death ; the mortality rate was 80–90 per cent.

Limited investigations only were carried out, but the condition was reproduced in monkeys and sheep. The results indicate that the ætiological agent was a virus, which was distinct from that of either poliomyelitis or epidemic encephalitis.

Herpes Zoster or Shingles

Herpes zoster is acute infection characterized by a vesicular dermal eruption with pain around the infected region. The infectivity is low ; small outbreaks have been reported, but they are rare. The vesicular eruption is frequently unilateral and is often preceded by local hyperæsthesia, itching or pain, which is referable to one or more nerve-roots corresponding to the level and side of the eruption. Perivascular and diffuse mononuclear infiltrations are present in the posterior root ganglia and the posterior horns of the spinal cord.

The ætiology is uncertain, but the indications are that the causative agent of zoster is a virus, identical with that responsible for varicella, but having no relationship with that of herpes simplex. Laboratory animals are refractory, but the lesions have been reproduced in children by the inoculation of the vesicular fluid, in which elementary bodies have been demonstrated. By means of the complement-fixation test it has been found that the serum of individuals recovered from an attack of varicella reacted equally well with antigens prepared from both zoster and varicellar lesions. The inoculation of susceptible children with zoster material resulted in the development of chicken-pox, whereas in children immune to chicken-pox no lesions developed. Although there appears to be a close relationship between these two viruses, there is a marked difference in their individual infectivity. The varicella virus is extremely infectious and attacks children, while the zoster virus has a low infectivity and usually attacks adults.

Varicella or Chicken-pox

Chicken-pox is a highly contagious disease frequently found in children and characterized by a cutaneous eruption. The condition is usually mild, complete recovery being the rule.

The identity of the ætiological agent with that causing herpes zoster has been discussed above. Nuclear inclusion bodies have been demonstrated in the epidermal and endothelial cells of the corium. The condition has been reproduced in children by the injection of the bacteriologically sterile vesicular fluid obtained from the specific lesions. Lesions with intranuclear inclusions have been produced by the intratesticular injection of the ground-up lesions into vervet monkeys.

A high degree of immunity follows an attack of chicken-pox ; convalescent serum has consequently been suggested as a possible therapeutic measure.

Acute Coryza or the Common Cold

Acute coryza is an acute infectious disease affecting the mucous membrane of the upper respiratory tract. The symptoms are relatively trivial, but they give rise to much local discomfort.

The condition is widely distributed, but although the lesions are readily accessible its ætiology remains uncertain. Numerous attempts have been made to incriminate a particular bacterium, but these have generally been unsuccessful. Very discordant results have been obtained by different workers, and there is no evidence to indicate that any one organism is the sole primary agent of the disease. It has been suggested that the common cold is not a single bacterial entity, but may be produced by any pathogenic member of the normal naso-pharyngeal flora, particularly *Strept. hæmolyticus*, *H. influenzæ* and the pneumococcus.

Other workers consider that a virus is primarily responsible and that the various complications, such as purulent discharges and antrum infections, are due to a secondary bacterial activity. Dochez and his colleagues claim to have reproduced the condition in series in chimpanzees and human volunteers by means of the filtered naso-pharyngeal washings of cases ; after cultivation of the virus *in vitro* similar results were also obtained. These results suggest that a filterable virus is a causative agent of the common cold, but whether it is the only agent responsible for this condition is still unsolved.

Mixed vaccines, containing the bacteria associated with respiratory catarrhs, have been widely employed in the prophylaxis of the common cold, and while the incidence has not been greatly influenced, it is claimed that the duration of the condition has been shortened and the onset of complications greatly reduced.

Infective Hepatitis

Infective hepatitis tends to be particularly prominent under war-time conditions. Sporadic cases and small outbreaks are usual in peace-time and the disease is often termed "catarrhal jaundice". Extensive outbreaks are encountered not infrequently among Service personnel; during the recent Middle East campaign the disease was widespread.

Infective hepatitis is characterized by general malaise, headache, nausea, vomiting and pyrexia with the appearance of jaundice some 7 days after the onset. The mortality rate is low. Limited histological studies have shown definite inflammatory changes in the liver cells.

The incubation period is long, 4–6 weeks in many cases. The mode of transmission is uncertain; some workers consider that the virus is spread by droplet infection, but attempts to isolate the virus have been mainly unsuccessful as laboratory animals have given negative results. In a few instances the disease has been reproduced in human volunteers by the intestinal route.

A closely related, if not identical, form of infective hepatitis has followed the administration of human serum or blood in the form of transfusions, convalescent serum or yellow fever vaccine; in these cases the blood was originally collected from individuals harbouring the virus. This disease is referred to as homologous serum hepatitis. The disease has also resulted from routine arsenical treatment in V.D. clinics; there is much evidence to indicate that infection in these cases was due to the contamination of the syringe with infected blood and incomplete sterilization before subsequent use.

Primary Atypical Pneumonia

Primary atypical pneumonia is a disease characterized by sudden onset, prostration, pyrexia with evidence of lung involvement which is mainly of an interstitial nature. This disease has been subjected to extensive investigations in America but the ætiological agent has not been definitely identified. Available evidence indicates that a virus is responsible but conclusive proof has not yet been obtained, although in a few instances the disease has been reproduced in human volunteers. Individuals with primary atypical pneumonia tend to form cold agglutinins which can be demonstrated by testing the serum of the patient against Group O cells at 4° C.; this agglutination is reversible at 37° C.

Verrucæ or Warts

Warts are benign papillary growths, the main clinical importance of which is cosmetic. They commence as a hyperkeratosis, and later there is a proliferation of the papillary tufts ; all the epithelial layers become thickened.

Little work has been carried out on this condition. The injection of both wart suspensions and their filtrates intradermally into human subjects has produced the characteristic lesions, which can be passed in series.

Little is known about the immunity to this condition ; some individuals appear to possess a natural immunity, while others are very susceptible.

Molluscum Contagiosum

Molluscum contagiosum is a contagious epithelial growth of benign nature. The condition is seen usually in children, the infection being transmitted by such articles as towels. The lesions are found generally about the face or genitalia, and consist of a nodule produced by local proliferation and swelling of the epithelial cells ; the central oval cells contain the so-called "molluscum bodies". Practically nothing is known about the immunity response.

C. POSSIBLE VIRUS DISEASES

Trachoma

Trachoma is a specific disease characterized by inflammation and hypertrophy of the conjunctiva. It is usually found in communities where hygienic conditions are poor.

The ætiology is rather obscure. Noguchi (1925) isolated a small Gram-negative bacillus from material removed from the eyes of American Indians suffering from trachoma. This he termed *Bact. granulosis*, and by sub-conjunctival inoculation into monkeys he claimed to have reproduced a condition resembling human trachoma. Noguchi concluded that this organism was the ætiological agent of the disease. Other workers have been unable to confirm these observations. While this failure may be due to the technical difficulties involved in the work, it has been suggested that the responsible agent is a filterable virus. Supporting this view is the presence of inclusion bodies in affected conjunctival epithelial cells. On the other hand, the actual existence of a filterable virus has never been demonstrated.

Acute Disseminated Encephalomyelitis

Acute disseminated encephalomyelitis is a disease of the central nervous system, and may occur either spontaneously or, more usually, in the wake of one of the exanthemata. It has received some prominence in recent years owing to its not infrequent occurrence in children and young adults following primary vaccination with calf-lymph. Cases have also been reported in association with measles, influenza, variola and during rabies prophylaxis. The primary infection usually runs a normal course and is settling down when nervous symptoms appear; myelitic and encephalitic forms may occur separately or combined. The mortality rate is about 30–60 per cent., but when recovery takes place it is usually complete; on autopsy changes are seen in the white matter and consist of diffuse perivascular infiltration with associated demyelination.

The ætiology is unknown. Attempts to reproduce the condition in experimental animals have proved futile. There is little to support the view that the organism responsible for the primary infection is also responsible for the nervous condition. It is generally thought that the nervous complications are due either to the activation of a latent virus or to the action of a toxin of unknown nature and origin.

Acute Rheumatism

For many years the association between the hæmolytic streptococcus and acute rheumatism has been recognized, but conclusive evidence that this organism is the actual causative agent of the disease has not been put forward. The ætiology remains obscure, but Schlesinger, Signy and Amies (1935) revived the virus theory following some interesting experiments. These workers demonstrated particles resembling elementary bodies in the pericardial and pleural exudates from cases of acute rheumatism. These bodies were agglutinated by the sera of patients suffering from, and successfully resisting, an acute rheumatic infection. It was concluded that there is a symbiotic relationship between *Strept. hœmolyticus* and the virus; the streptococcal infection lowers the general resistance of the host and the virus is so enabled to enter the body and assume active characters. Further work is, however, required before this theory can be accepted without reservation.

Hodgkin's Disease or Lymphadenoma

The ætiology of Hodgkin's disease is obscure. Gordon, however, obtained evidence suggesting the participation of a virus in the disease process. Suspensions of enlarged glands from cases of the disease produced a fatal meningo-encephalitis on intracerebral injection into the rabbit. It has not, however, been possible to pass the condition in series through these animals. The glands were found to be bacteriologically sterile, but by special staining methods small coccal bodies resembling elementary bodies were demonstrated. While these and other results may suggest the virus nature of the causative agent of lymphadenoma, the failure to transmit the nervous condition to rabbits in series also suggests the action of a neurotoxin. Further work is necessary before the ætiology can be considered solved. The rabbit test of Gordon has, however, proved a valuable diagnostic measure. It has been suggested that the encephalitic substance is derived from the eosinophil leucocytes, which are a prominent histological feature of the condition. It is pointed out that, when the histology of a gland is uncertain and eosinophils are scanty, the test is generally negative.

New Growths

The possibility that viruses might be concerned with the ætiology of tumours has long been appreciated, and much work has been focussed on this complex problem. Progress has undoubtedly been made, but evidence that viruses are in any way responsible for human tumours has not been obtained. It has, however, been demonstrated that certain tumours are caused by viruses ; these are a large group of fowl sarcomata, a papilloma of rabbits which tends to become malignant, and an adeno-carcinoma of the frog. In these cases it is considered that the virus enters the tumour-cells and provides a continuous stimulus which is responsible for the prolific multiplication of the cells. These tumours possess similar properties to other mammalian tumours in which a virus cannot be demonstrated. They are non-infective under natural conditions, occur sporadically and spread through the body by cell-metastasis. The origin of the virus, *i.e.*, whether exogenous or endogenous, and the mechanism of infection in these cases have not been determined. Nevertheless these observations are of great fundamental importance and have aroused considerable interest. As a result of these observations the virus theory has been used by some workers to explain all or most tumour phenomena, but, while this view should not be lightly dismissed, further work is necessary before it can be applied with any certitude to tumours other than those mentioned above.

CHAPTER XXXIII

THE RICKETTSIÆ

SMALL rod-shaped bodies, frequently present in the alimentary tract of arthropods, have been found in association with certain diseases of man and animals. These bodies were designated *Rickettsia* by Da Rocha-Lima (1916) in honour of Ricketts, who first described them in 1909 in cases of Rocky Mountain spotted fever. Many species have since been observed; at least seven of these have been found in association with disease in man; some thirty-nine, apparently non-pathogenic to mammalia, have been found in arthropods.

The *Rickettsiæ* are generally accepted as minute forms of bacteria representing a stage of biological evolution intermediary between the viruses and the bacteria.

General Properties. The *Rickettsiæ* are minute bodies of an average size, $0{\cdot}5 \times 0{\cdot}3\mu$. Pleomorphism is a common feature, diplococcal and relatively long forms of 2μ may be seen. The majority of the species are non-motile. These bodies are difficult to stain and when stained their outline is somewhat indefinite; they are Gram-negative. Giemsa is one of the most suitable methods of staining; the bodies are purplish and bipolar staining is a common feature.

The *Rickettsiæ* do not grow on the ordinary bacteriological media; special media, such as agar covered with chick embryonic tissue or the yolk-sac of the chick embryo, have given good results. The most satisfactory method of isolating the rickettsiæ is the inoculation of susceptible laboratory animals, of which the guinea-pig is the most valuable. Blood from suspected cases is generally injected by the intraperitoneal route; a fever with a temperature of about 104° F. is produced in 5–12 days, the animal should be killed during this phase and the rickettsiæ are found in large numbers in the spleen and brain.

The *Rickettsiæ* do not pass through the usual bacterial filters, and their resistance to bactericidal agents is similar to that exhibited by the vegetative bacteria.

The serological behaviour of these bodies has not been studied in detail; agglutinins, either acting against the specific rickettsia or giving the Weil-Felix reaction, have been produced by the injection of rabbits with the infected excreta of lice. Cross-immunity tests in guinea-pigs, rabbits and monkeys are also being used in some cases with successful results.

The *Rickettsiæ* have been found in close association with the following diseases of man of which they are considered by most bacteriologists to be the ætiological agents (Table XXXI.).

TABLE XXXI

Human Rickettsial Diseases

Disease	Rickettsia	Insect Vector	Weil-Felix Reaction	
			OX 19	OXK
Epidemic or exanthematic typhus.	*R. prowazeki.*	Louse.	+++	(+)
Endemic or murine typhus	*R. mooseri*	Rat flea	+++	(+)
Scrub typhus (Tsutsugamushi fever).	*R. orientalis* (*nipponica*).	Mite larva	0	+++
Rocky Mountain spotted fever	*R. rickettsi*	Tick.	++	++
Fièvre boutonneuse	*R. rickettsi* (*conori*).	Dog tick	++	++
Trench fever	*R. quintana*	Louse	0	0
Q fever	*R. burneti*	Tick.	0	0

Typhus Fever

Typhus fever, known also as gaol fever, camp fever, endemic or urban typhus, is a disease associated with insanitary conditions, overcrowding, poverty or famine and at one time was widespread, epidemics being frequently encountered. At present it is uncommon in most civilized communities where marked improvement in sanitation has taken place; in this country it is mainly of historic interest although cases occurred among British troops in Africa and the Far East during the past war. Typhus fever has a world-wide distribution and is most prevalent in the colder months of the year, when people tend to crowd together.

The classical or epidemic disease is transmitted by the agency of the louse, which becomes infected by sucking the blood of patients. The period of infectivity in lice varies from 2–10 days. Man is infected either by the bite of an infected louse or through wounds produced by scratching skin contaminated by the excreta of the insect; there is an incubation period of from 5–20 days, with an average of 14 days. The onset is sudden and is characterized by fever, headache, vomiting, and on the fourth or fifth day by the appearance of a generalized macular or hæmorrhagic rash. After 14 days the temperature usually falls by crisis and convalescence, which should be prolonged, ensues. The mortality rate varies and depends on the age and condition of the patient ; it may be as low as 2 per cent. or as high as 30 per cent. A high degree of immunity follows an attack.

Murine typhus, the ætiological agent of which is *R. mooseri*, is a similar but milder disease, which can be differentiated from epidemic or exanthematic typhus by serological tests with rickettsial suspensions. *R. mooseri* is antigenically related to *R. prowazeki* and is usually transmitted to man from rodents, particularly the rat, by the flea, *Xenopsylla cheopis*.

Bacteriology. *R. prowazeki* is generally accepted as the ætiological agent of epidemic typhus fever. This relationship was first described by Da Rocha-Lima, who observed large numbers of the organisms in the intestinal tract and the lining epithelial cells in the majority of lice collected from typhus patients. They were not found in lice obtained from healthy individuals. It was later shown that lice containing *R. prowazeki* were able to produce infection in guinea-pigs and monkeys ; Rickettsia-free lice were not infective. The demonstration of the organisms in human patients is difficult, but they have been found in various vascular lesions.

Monkeys and guinea-pigs can be infected by the subcutaneous inoculation of blood collected from cases during the febrile period. The condition is similar to the spontaneous human disease. In some cases the guinea-pig, while becoming infective, does not give any reaction ; to this condition Nicolle and his colleagues have given the term " masked typhus ".

Diagnosis. The demonstration of *R. prowazeki*, either in lice caught on typhus cases or in human lesions, is not widely employed in practice as a diagnostic measure owing to technical difficulties.

A serological test discovered by Weil and Felix in 1916, and referred to as the Weil-Felix reaction, is now widely used and gives constant and reliable results. The reaction is an agglutination by the serum of typhus patients of a strain of *Proteus* organism isolated from the urine of a case of typhus fever. Several strains of this organism were isolated, and the one giving the most specific results was termed *Proteus OX* 19. The agglutinins appear in the patient's serum on the fourth or fifth day, increase until the second or third week, and then decrease until by the end of the fifth month they may have disappeared. Titres as high as 1/50,000 are obtained during the febrile stages, and the agglutination is invariably of the O type.

The constancy of this reaction raised the question of the relationship of the Proteus OX 19 strains to typhus fever. Attempts to incriminate them as the ætiological agent have been, however, unsuccessful. The organisms could only be isolated from a few cases ; they did not produce either the experimental infection or immunity in guinea-pigs to infection with rickettsiæ. While some workers consider that the *Rickettsiæ* represent a phase in the life cycle of the *Proteus* strains, others have postulated the view that the agglutination with Proteus organisms is due to these organisms having antigens in common with the rickettsiæ.

Agglutination or complement-fixation tests with specific rickettsial suspensions can be used to establish the identity of the infecting organism.

Prophylaxis. The prevention of typhus fever depends mainly on the control and eradication of the louse, with attention to general hygienic conditions, personal hygiene and cleanliness. In the recent Great War dusting with insecticides such as D.D.T. was widely used with great success.

Active immunization, produced by the inoculation of living, attenuated or killed rickettsiæ, has been practised with encouraging results. The main difficulty originally was to obtain an adequate supply of the rickettsiæ but this has been overcome by the use of yolk-sac cultures or the lungs of infected rabbits or mice. The duration of the resulting immunity is uncertain and frequent dosage is necessary.

Trench Fever

Trench fever was a disease which appeared among the troops during the first Great War and disappeared at its termination. It appeared in many war zones and had a high morbidity rate. Trench fever was characterized by recurrent temperature, sweating and polyuria ; recovery was usually complete, the disease was never fatal. The condition was not diagnosed for some time after its appearance. McNee, Renshaw and Brunt (1916) succeeded in reproducing the disease in man by the injection of the citrated blood of a patient. The *rôle* played by lice was suspected by clinical and epidemiological observations, and their capacity to transmit the disease from man to man was determined. *Rickettsiæ* were found in the excreta of lice fed on cases and were called *R. quintana*. They were not detected in lice fed on healthy individuals. They have not been demonstrated in material obtained from the human disease. The ætiological relationship of *R. quintana* to trench fever has not therefore been conclusively proved.

Rocky Mountain Spotted Fever

Rocky Mountain spotted fever is a condition resembling typhus fever and found only in certain districts of America. It is the disease in which the *Rickettsiæ* were first detected by Ricketts in 1909. Ricketts had previously reproduced the disease in healthy individuals and guinea-pigs by the injection of the defibrinated blood of cases. The organisms were later found in ticks that had been feeding on cases.

Fièvre Boutonneuse

This is a mild disease found mainly in Tunisia and characterized by the appearance of a local nodule at the site of the insect bite. The ætiological agent is a rickettsia which appears to be identical with *R. rickettsi*, the causative agent of Rocky Mountain spotted fever. It is transmitted from dogs by the dog tick.

Scrub Typhus

This disease is identical with the Japanese River or Tsutsugamushi fever, which is transmitted by a larval mite, *Trombicula deliensis*; the main reservoir of the organism is the rat. A positive Weil-Felix reaction with OXK strains of Proteus is obtained in both conditions. The disease has a low mortality rate, although in some districts this may be as high as 30 per cent. It was an important disease among troops in the Far East (S.E.A.C.).

Q Fever

Q fever is a disease recently described in Australia among workers in dairies and abattoirs characterized by an acute illness of 7–24 days' duration. The mortality rate is nil. Agglutination is not given with *Proteus* organisms but a rickettsia has been isolated from cases and is known as *R. burneti*.

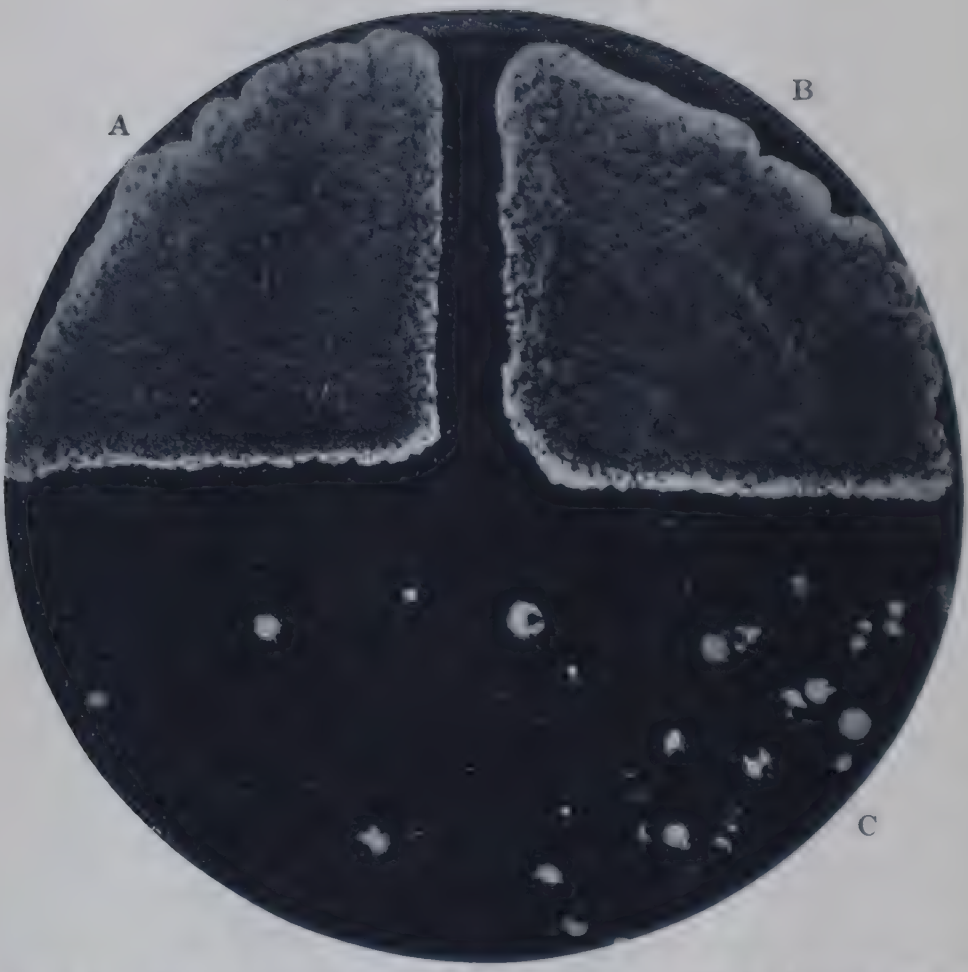
CHAPTER XXXIV

BACTERIOPHAGE

A PHENOMENON of great fundamental biological importanc
that described first by Twort in 1915, and later, in 1917,
D'Herelle, and frequently referred to as the "Twort-D'Her
phenomenon". Twort, during some experiments with glyc
nated calf-lymph, noted that, after cultivation on agar, so
colonies of a white micrococcus developed; these on furt
incubation became glassy and transparent. On microscop
examination it was found that these glassy areas did not cont
any micrococci but were composed mainly of minute, indefi
granules. The same lytic action resulted when similar int
colonies were inoculated with the glassy material, and in t
way the change was transmitted indefinitely. It was found
take place more actively in young multiplying organisms tha
old ones, while there was no obvious effect on killed organis
The responsible agent readily passed through bacterial filt
The nature of this reaction was not determined, although sev
possible theories were expressed. Further work was preven
by the exigencies of the war.

D'Herelle (1917) observed changes of a comparable nature
cultures grown in a fluid medium. He found that when fa
from dysentery cases were emulsified in broth and filtered,
addition of a few drops of the filtrate to young broth culture
Bact. shigæ arrested the growth of the organisms, which beca
lysed. This phenomenon was carried on indefinitely by filte
the lysed culture and adding the filtrate to fresh young cul
of the same organism. D'Herelle carried out extensive invest
tions to determine the nature of the reaction, which he conclu
was different from that described earlier by Twort; he conside
that it was due to the activity of a filterable virus, which
termed *Bacteriophagum intestinale*. These observations have b
confirmed and elaborated by numerous investigators, most
whom consider, however, that the phenomena described indep
dently by Twort and D'Herelle are identical. Although the vi
theory is not universally accepted, the responsible agen

PLATE VI



A. & B. Heavy inoculation of *Bact. typhosum* with its bacteriophage, showing numerous clear circular areas—"plaques" or "taches vierges."

C. Light inoculation of *Bact. flexneri* with bacteriophage, showing "nibbled" or "bitten" colonies.

[*To face p.* 431.

generally termed either *bacteriophage* or simply *phage*, and the phenomenon *bacteriophagy*.

General Properties. Bacteriophage has the characteristic property of clearing young broth cultures, either temporarily or permanently, and of forming clear areas in young cultures on solid media. These clear zones have been styled " taches vierges ", plaques or colonies ; they are considered by D'Herelle to represent colonies of the virus. Single, discrete, bacterial colonies usually present a nibbled appearance (see Plate VI.). There is no such effect on killed organisms, which, however, can adsorb the phage. The action of a phage may not be confined to one particular organism ; when several organisms are acted upon by a phage, the action is unequal and tends to be more marked against certain organisms than others. This multiplicity of action by a phage is considered in many cases to be mainly due to the presence of common O factors in the antigenic structure of different bacterial species acted upon ; in some cases, however, a phage may act on quite unrelated species. It is possible to enhance the activity of a phage for an organism by serial passage, and there is no doubt that the phages exhibit marked adaptability. Many organisms, particularly members of the *Bacterium* genus, have been found to be susceptible to phage action.

The addition of a phage to a young broth culture may exceptionally result in complete and permanent clearing, the culture becoming sterile. Usually there is an initial clearing followed by the reappearance of turbidity, due to the fact that some organisms are not lysed and multiply in spite of the presence of the active phage. On subculture these organisms are frequently resistant to the phage. Moreover, these secondary cultures of resistant organisms may contain bacteriophage ; the resistant organisms are thus lysogenic without being themselves affected by the bacteriophage. The exact nature of these resistant strains is uncertain, but they frequently exhibit marked differences from the parent strain and tend to resemble the R or rough variants and also mucoid variants.

Phages are particulate, filterable particles ; by means of ultra-filtration through gradocol membranes variations from 0·008 to 0·075μ have been noted in the size of the individual phages. There is also considerable variation in activity ; in some cases the lytic substance is active in very high dilutions, *e.g.*, 1 in 10^6. Phages are, however, quite uniform in the resistance offered to destructive agents ; resistance is offered to drying, glycerine, alcohol, phenol (1 per cent.) and freezing, but they are readily destroyed by ultra-violet rays and heat over 70° C. Phage contained in sealed tubes and kept in the dark at room-temperature retains its activity for many years.

There appears to be little doubt that phages possess antigenic properties independent of the other substances present in the phage filtrates. Antiphage sera can be readily prepared in rabbits. The mechanism of the action of antiserum on bacteriophage is unknown; some workers have suggested that it is an adsorption process.

Bacteriophage is widely distributed in nature. It has been isolated with great frequency from the fæces of man and animals, sewage, water and soil. It has also been obtained from samples of pus, and some workers claim that it can be generated spontaneously by serial cultivation of an organism in its own broth filtrate.

The Nature of Bacteriophage. The nature of the lytic principle has not been determined. Many divergent views have been expressed at various times, but only three appear to be possibilities: (*a*) virus theory, (*b*) the enzyme theory, and (*c*) the theory of hereditary factors.

(*a*) The virus theory has many supporters. The adherents of this school consider that the adaptability and the capacity to multiply only in the presence of young living cells indicate the living nature of the active substance and an analogy to the viruses. While, however, some agree with D'Herelle that there is only one phage, which exhibits an almost infinite capacity for modification and variation, the majority of workers are of the opinion that there are many separate and distinct phages.

Support for the virus theory has been obtained from the use of the electron microscope. Limited observations indicate that phages have a definite structure and, in the case of certain coliform and dysentery bacilli, possess a head and a tail.

(*b*) The enzyme theory holds that the lytic substance is not living, but is an autolytic enzyme liberated by the disintegration of the bacterial cell and able to induce the lysis of other susceptible organisms, which in their turn set free more enzymes of the same nature.

(*c*) The hereditary theory postulates that the lytic principles are stable elements of cellular origin and are hereditary factors in the Mendelian sense.

Therapeutic Application. Many claims have been made of the successful application of phage in the treatment of infections, particularly in the case of certain intestinal, cutaneous and urinary diseases. In many instances there has been little evidence to support these claims, which the majority of workers have been unable to confirm. There is no doubt that an active phage readily lyses susceptible bacteria *in vitro*, but, while this suggests the phage as an admirable therapeutic agent, a marked difference is unfortunately found in its *in vivo* action. Two factors are mainly responsible for this : (1) in the tissues there is much extraneous tissue which readily adsorbs the phage, which is thereby inactivated, and (2) the presence of resistant strains of the organism, which are unaffected by the phage. Consequently, in view of the limitations of our knowledge of the bacteriophage, it is as yet of little value as a therapeutic agent of general application.

CHAPTER XXXV

THE BACTERIOLOGY OF WATER, MILK AND SHELLFISH

WATER

WATER is the main constituent of the body and is consequently a necessary article of diet. Water for drinking purposes is obtained from various sources and is divided according to its origin into two main types—(*a*) surface and (*b*) deep.

(*a*) Surface waters comprise those collected at or near the surface of the ground and include rain, lakes, rivers and reservoirs. This type is exposed to contamination from many sources and invariably contains bacteria.

(*b*) Deep waters are collected from an underground source, such as wells and springs. The water undergoes a process of filtration during percolation through the soil and is usually bacteria-free at the source.

Surface waters are the main source of drinking-water on account of their accessibility. Such water, as previously stated, always contains bacteria, the common saprophytes being invariably present. Lakes and rivers may be heavily contaminated, as they receive the drainage from the neighbouring land and are sometimes used for the disposal of sewage. When sewage is disposed of by other means the sewage and water systems may run close together and fractures and leakages in the pipes have resulted in contamination of the water supply. It therefore follows that the presence of pathogenic organisms is not uncommon in this type of water. As in some districts it is consumed in an untreated state, it may be responsible for serious outbreaks of infections, the most important of which are those involving the intestinal tract, especially enteric fever, dysentery and cholera. Many such epidemics have been reported in which the drinking-water has been the vehicle of infection. The water supply of large towns is either brought from remote regions many miles away or is collected from local rivers, and is strictly controlled. In rural districts, however, drinking water is frequently obtained from shallow wells situated close to dwelling-houses and exposed to contamination ; these supplies do not usually conform with the accepted bacteriological standards for drinking water.

There is thus a definite necessity for a rigid control of the composition of drinking water. This is carried out by attention not only to the bacterial content, but also to the physical appearance and the chemical analysis.

The bacteriological examination constitutes an important part of the control of water, but in view of the inherent difficulties the methods available are far from satisfactory. A final judgment of the potability of a water should never be given from the bacteriological examination alone ; in all cases detailed information of the topography of the drainage area should be available. It is also important to note that a single bacteriological investigation, unless quite unsatisfactory, is of very limited value. The detection of the important pathogenic organisms is difficult and often impossible, and this consequently does not form part of the routine test, which is devised to determine (1) the total number of living bacteria and (2) the presence of coliform organisms.

A representative sample of the water is collected in a sterile, stoppered, 200 ml. bottle, placed in a special ice-box and submitted to the laboratory without delay. Two or three successive tenfold dilutions of the sample are then made in sterile water, using a fresh sterile pipette for each dilution and mixing well at each stage. Using a fresh pipette, 1 ml. of each dilution is added to a Petri dish with either melted agar or gelatin and the contents well mixed ; the number of dishes required depends largely on the nature of the original sample. On cooling, the gelatin plates are incubated in an upright position at 22° C. and the agar plates, inverted, at 37° C. After 2 days the agar plates are counted and after 3 days, the gelatin plates. The results are averaged and expressed as so many bacteria per cubic centimetre or millilitre of the original sample. Most of the bacteria growing on the plates incubated at 22° C. are saprophytes and the information obtained from this test is of very limited value, being mainly an indication of the suitability of the water for bacterial growth. This test is consequently omitted in many routine examinations.

The examination for coliforms is usually carried out by adding various amounts of the sample, *e.g.*, 50 ml., 10 ml. and 1 ml., to tubes of fluid MacConkey's medium, which contains lactose, bile salts and neutral red. These are incubated at 37° C. for 2 days ; subcultures are made on solid MacConkey's medium from the highest dilutions showing acid and gas formation. Two or three lactose-fermenting colonies are then selected from each plate and subjected to tests, which include (1) indole production, (2) methyl-red, (3) Voges-Proskauer, (4) growth on uric acid medium, and (5) growth on citrate medium.

The coliforms have been divided into two main groups: (1) *Bact. coli* group, which tend to be indole +, M.R. +, V.P. −, uric acid −, citrate −; and (2) *Bact. aerogenes* group, which are indole ±, M.R. −, V.P. +, uric acid +, citrate +. There is another group containing coliforms not conforming with either of these standards; these are termed *intermediates*.

A simple test to differentiate fæcal from non-fæcal coliforms has recently been introduced and is based on the production of acid and gas in MacConkey broth at 44° C. by fæcal coliforms. Sub-cultures into MacConkey broth are made from the original MacConkey broth tubes showing acid and gas; the sub-cultures are incubated in a water-bath at 44° C.—the temperature must be carefully controlled as variation may lead to irregular results. After 24 hours' incubation tubes showing acid and gas are accepted as containing fæcal coliforms. This test has given satisfactory results and is considered to be practically specific for fæcal coliforms.

The results of the coliform tests have previously been given according to the following formula: present in 100 ml., absent in 10 ml. Many objections have been raised against this system and a new scheme, based on the laws of probability, has been introduced. Tables giving the probable number of coliforms per 100 ml. have been prepared according to the number of positive results given by the various quantities of water used in the test and the final report is a figure giving the probable number of coliform organisms per 100 ml. of the water under examination

The presence of members of the *Bact. coli* group, or fæcal coliforms as they are generally termed, is of the greatest significance as this is indicative of recent fæcal pollution. Organisms of the *Bact. aerogenes* group, or the non-fæcal coliforms, are of less significance as they are considered to be introduced mainly from the local soil and vegetation; they have, however, been isolated on certain occasions from the fæces of man and animals and this division is therefore not strictly correct.

The interpretation of the results is frequently difficult, as they are only of relative value and no officially accepted standards are available for comparison. The results of a single examination are generally of doubtful significance, and a tentative report only can be given in such cases. When, however, repeated examinations of one water are made, rough standards can be fixed and any marked deviations from these should be viewed with suspicion.

The following points give useful information in assessing the potability of a water. The gelatin count indicates the number of bacteria present and the suitability of the water for bacterial growth; the majority of these organisms are harmless saprophytes normally present in soil and in the air. The agar count gives more definite information of the number of organisms coming from human or animal sources; many of the common saprophytes do not grow readily at 37° C. There should therefore be ordinarily a much higher gelatin count than an agar count, which should be low, *i.e.*, below 50–100 per millilitre.

The presence of *Bact. coli* is of greatest practical importance, as this is the most direct available evidence of fæcal pollution. The following working rule is adopted: the greater the fæcal pollution the greater the chance of the presence of pathogenic intestinal organisms. Therefore water with a relatively high *Bact. coli* count, ten or more per 100 ml., is considered to be unsuitable for drinking purposes, while a count of 3 to 10 per 100 ml. is regarded as suspicious. Further tests to indicate fæcal pollution are the examination for *Cl. welchii* and *Strept. fæcalis*, but these are not usually performed in routine work.

An examination for the presence of *Bact. typhosum* or *V. choleræ* is sometimes required, but this is rarely successful. Many methods have been tried, the most suitable being the concentration of the bacteria by centrifuging and seeding on special media, such as Wilson and Blair's, or adding large quantities of the water to equal quantities of a special broth with or without inhibitory substances, such as brilliant green.

Water from contaminated sources can be rendered potable by various processes which destroy or remove bacteria. Those commonly employed are storage, sand-filtration, chemical treatment, *e.g.*, chlorination, and treatment with ultra-violet light. The success of these measures has been reported in numerous public health records, and is indicated by the rarity of water-borne epidemics in civilized communities at the present time. In the household the purification of water is carried out either by boiling or by the use of a bacterial filter.

Swimming-bath Water. The great increase in the popularity of bathing during recent years has directed attention to the bacteriology of swimming-bath water. There is little doubt that the untreated water of bathing pools may become a suitable medium for bacterial multiplication, and during hot weather when the baths are crowded there are numerous opportunities for contamination with human excreta. Diseases stated with some justification to have been contracted in swimming pools include otitis media, infections of the upper respiratory tract and enteric fever. It is therefore natural that the condition of the water should be subjected to some control; the measures taken in this direction include continuous filtration of the water, treatment of the water with chemicals, such as ozone or chlorine (0·3 parts per million), and repeated bacteriological examinations. There are unfortunately no accepted standards for the bacterial content of such waters. It is usual to carry out an examination similar to that used for drinking-water, *i.e.*, a determination of the bacterial content and a coliform test. The interpretation of the results presents many difficulties and the bacteriologist can only suggest that the condition of the water is either satisfactory or not.

MILK

Milk is an extremely valuable food; it contains proteins, fat, carbohydrates, inorganic salts and is rich in vitamins. It is the sole supply of food for infants and forms a large portion of the diet of children. Milk from animal sources is mainly employed for this purpose; in many countries it is obtained from the cow, but in some cases reindeer, goats or mares are used. It is therefore essential that the standard of milk should be rigorously controlled, as there is much opportunity for it to become unsuitable for human consumption. Four methods of examination are employed for this purpose: (1) physical—taste, odour, appearance, etc.; (2) chemical—content of fat and total solids; (3) sanitary—veterinary inspection; and (4) bacteriological. Of these only the bacteriological examination need be considered here.

The *bacteriology* of milk is a complex problem. Milk is an excellent medium for bacterial growth, and as the possibilities of introducing bacteria into milk are great, they are frequently numerous and varied. Bacteria may be introduced from four main sources: (1) the udder and fæces of the cow, (2) the milker, (3) the air, and (4) unclean utensils. The number present is dependent also on the time between collection and examination of the sample and the temperature at which it has been kept. During the warm months bacterial multiplication readily takes place at the room-temperature. Milk contains some bactericidal substances, but these are of little value in restricting and controlling bacterial growth.

The bacterial content of milk is usually extremely varied and may be conveniently divided into two main groups : (1) normal flora containing the saprophytic organisms naturally occurring in milk and (2) organisms pathogenic to man.

(1) **Normal Flora.** Members of this group do not produce disease in man, but by their activity they may alter the physical appearance of the milk and so render it unsuitable for consumption. The following sub-groups are frequently present : (1) acid-forming bacteria, *e.g.*, *Strept. fæcalis* and the *Lactobacilli*, these ferment lactose to produce lactic acid, which converts caseinogen into the insoluble casein ; sour milk with curdling is produced ; (2) alkali-forming bacteria, *e.g.*, *Bact. alkaligenes*, which saponify the fat ; (3) proteolytic organisms, *e.g.*, *B. subtilis*, *Proteus* and *Staphylococci*, which give rise to whey formation through the digestion of the casein by " casease " ; (4) gas-forming bacteria, *e.g.*, *Cl. welchii* and *Bact. coli.* ; and (5) inert organisms, including the air cocci.

(2) **Human Pathogens.** Many important human diseases are due to the presence in milk of pathogenic bacteria ; these diseases are seen most frequently in children, who are the greatest consumers of this commodity. There are two chief sources for these organisms :

(*a*) Infections in the milker or other individuals handling the milk.

(*b*) Infection in the cow.

(*a*) Pathogenic organisms may be implanted in the milk during either the milking process or the subsequent handling to which milk is subjected. The usual mode of introduction is by coughing or sneezing ; several outbreaks have been reported in which milk, contaminated in this manner, has been the vehicle of infection. The diseases so transmitted have been sore-throat, scarlet fever, diphtheria, tuberculosis and possibly poliomyelitis. In other instances infections of intestinal origin, such as enteric fever, have been transmitted by the agency of milk. When such outbreaks have occurred subsequent examination has usually shown that some individual handling the milk was a carrier of the responsible organism. It is an important function of public health administration to ensure that carriers of pathogenic bacteria are not concerned with the handling of milk or other foods.

(*b*) The majority of human diseases produced by the consumption of milk are due to the presence of infection in the cow from which the milk is collected. Several important animal pathogens are also able to produce disease in man ; the most important of these organisms are the tubercle bacillus, *Br. abortus*, some strains of *Strept. hæmolyticus* and members of the food-poisoning group, particularly *Bact. enteritidis*.

The eradication of bovine tuberculosis is one of the most important social and economic problems of the present time. In this country it is estimated that approximately 10 per cent. of all samples of mixed milk contain tubercle bacilli and that some 2–5 per cent. of the cattle are infective ; all, however, may not show definite signs of infection. The general consumption of untreated milk is thus both a potential and real danger to the community, particularly to children, in whom infection with the bovine type of the tubercle bacillus is usually manifested by lesions in the glands, abdomen and bones. The introduction of various prophylactic measures, such as the inspection of cows, tuberculin testing, bacteriological examinations and pasteurization, has reduced the incidence of these infections. Owing to the lack of general application, these measures have had only a limited effect, as in 1931 deaths from infection with the bovine tubercle bacilli numbered no less than 1,716.

Bacteriological Examination. This involves (1) an estimation of the number of living bacteria present and (2) the detection of pathogenic organisms.

(1) The number of bacteria in a given sample of milk is dependent on many variable factors. The method of collection, the temperature at which kept, and the time between collection and examination are all factors greatly influencing the bacterial content. Until the investigation by the Medical Research Council (1935) the methods of examination used in this country were similar to those employed for water, viz., a total count by dilution and plating together with a coliform estimation.

The experimental error involved in these tests was found to be too great to ensure their reliability and accuracy. There were also other factors rendering these tests misleading. The count obtained by the plating method was not necessarily complete, as all the organisms originally present in the sample do not multiply under the conditions of growth; also a colony does not invariably indicate the growth of one organism, but might be produced by an aggregation of bacteria. In the case of the coliform test it was often found that non-fæcal coliforms only were present, and, as this did not indicate fæcal contamination, their presence was therefore of little pathogenic significance.

In view of these findings it was considered that the old method of testing milk was unsuitable; it was not only expensive and tedious, but it also required experienced workers in whose hands the results were far from being a reliable index of the potability of a milk sample. It was consequently considered that the system of grading milk was unfair, as the criteria laid down in the different grades were so easily subjected to variation. The following bacteriological standards had been accepted for the different grades:

"Certified Milk" must not contain more than 30,000 organisms per cubic centimetre, and must not contain *Bact. coli* in 1/10 c.c.

"Grade A Milk" must not contain more than 200,000 organisms per cubic centimetre and *Bact. coli* in 1/100 c.c.

"Pasteurized Milk" must not contain more than 100,000 organisms per cubic centimetre; a "coli" test was not laid down.

It is seen that no standards are laid down for ungraded milk, which is consumed in much greater quantities and by many more individuals than the graded forms.

Other methods of examination were tested, and it was shown that satisfactory results could be obtained by means of a modification of the methylene-blue reduction test, which was both simple and economical.

Methylene-blue Reduction Test. In this test 10 ml. of the milk are placed in special sterilized graded test-tubes and 1 ml. of a standard methylene-blue solution is carefully added, giving a final methylene-blue concentration of 1/300,000. The tubes are plugged with sterilized rubber corks, placed in a water-bath at 37° C. (for pasteurized milk a series may also be incubated at 55° or 63° C.), and inverted once every half hour to ensure a homogeneous distribution of the fat-globules and bacteria. The end-point is taken when the dye is completely decolorized, or decolorized to within 5 mm. of the surface. In milk there are two main reducing systems: (1) present in "sterile milk", and (2) present in milk in which bacteria are actively multiplying

The first system appears to result from enzyme activity and is partly inactivated by pasteurization. While variations in this activity are displayed by single milks, mixed samples tend to exhibit little variation. This factor consequently does not interfere with the application of the test to the examination of mixed milks, in which the reduction test is regarded as a measure of the total bacterial metabolism. The more living bacteria present in a sample the shorter is the reduction time under the conditions of the test.

The conclusions of the investigation were that all liquid milk for human consumption should reach a standard corresponding approximately to the present standard for Grade A milk. With the modified methylene-blue reduction test 75 per cent. of samples of morning milk, left at atmospheric temperature for 12 hours after milking and subsequently refrigerated overnight, should have a reduction time of over 5½ hours in the summer and of over 6½ hours in the winter. The present system of grading milk was not recommended.

New standards are described in various Milk (Special Designations) Orders and Regulations, 1936–43. Four main grades are accepted : (1) tuberculin-tested, (2) accredited, (3) pasteurized and (4) heat-treated. The accredited and tuberculin-tested milks correspond closely to the old Grade A standard, and the methylene-blue reduction time should not be less than 4½ hours in summer and 5½ hours in winter. It is, however, important to note that it is not necessary for milk producers to conform to the standards, which are intended mainly to indicate special qualities. The bulk of the milk consumed by the public is still ungraded and consequently is a potential source of danger ; for this reason efficient pasteurization still constitutes a safeguard of paramount importance.

Milk (Special Designations) Regulations, 1946, which came into operation on March 1st, require that pasteurized milk and tuberculin-tested (pasteurized) milks shall comply with prescribed phosphatase and methylene-blue tests ; plates counts are no longer required.

(2) Milk is frequently examined for the presence of human pathogens, particularly the tubercle bacillus. Several methods may be used to detect tubercle bacilli in milk—microscopy, culture and pathogenicity tests.

Pathogenicity tests have held the pride of place for many years The usual procedure is to take two samples of the milk and centrifuge in special Delépine centrifuge tubes, by means of which the deposit can be readily obtained from the supernatant fluid. The deposit, 2 ml., is injected into the groin of a guinea-pig, *i.e.*, two animals (A and B) are used for each sample of milk. These animals are carefully labelled and kept under observation ; the A pig is killed after 3 weeks and the B pig at the end of 5 weeks. An autopsy is made, and any tuberculous lesions are noted and carefully examined for the presence of the tubercle bacillus.

This method is far from satisfactory, as it is costly and requires several weeks before the result is known. Many attempts have been made to replace it by a simpler test. In recent years it has been claimed that both microscopical and cultural methods can give equally satisfactory results. For the cultural method a modification of Lowenstein's method is employed. The milk deposit is treated with either NaOH or H_2SO_4, neutralized and seeded on to a selective medium. After incubation for about 3 weeks it is stated that not only can the presence of the tubercle bacillus be detected, but also its type can be determined.

In the microscopical method the milk deposit is stained by the Ziehl-Neelsen method and is examined by the low-power magnification for the presence of *cell groups*, which are collections of endothelial cells. When these are found the field is examined by the oil-immersion lens for tubercle bacilli. It is stated that the tubercle bacilli are usually present in or around these cells.

While these tests appear to yield useful results, they do not appear to be sufficiently reliable entirely to replace the more costly pathogenicity tests.

The examination of milk for the presence of *Br. abortus* is becoming increasingly important. Investigations may be carried out by culture, serological methods or pathogenicity tests. In the pathogenicity test about 2–4 ml. of the sample are injected subcutaneously or intraperitoneally into a guinea-pig. The animal is killed at the end of 4–5 weeks ; blood and material from the spleen and regional lymph glands are seeded on liver-infusion agar and placed in an atmosphere containing 10 per cent. CO_2 at 37° C. A similar medium containing 1 in 100,000 gentian-violet and adjusted to pH 6·6 is used in the cultural method. Serological tests depend on the presence of agglutinins in the milk and are less reliable than the isolation of the organism.

The examination for other pathogens is more complicated, special technique is required and positive results are frequently difficult to obtain.

Pasteurization. The pathogenic organisms likely to be present in milk are non-sporing organisms and all are relatively easily destroyed by heat. For this reason the process of pasteurization is able to kill the pathogens and most of the other organisms found in milk without interference with its physical properties ; thermophilic organisms survive the process, but apart from exceptional circumstances they are of little significance. Pasteurization is usually carried out either by the " holding method " or the less satisfactory " flash " method. In the *holding method* the milk is kept at a temperature of 145° F. for 30 minutes, after which it is rapidly cooled ; this is the method officially recognized in this country. The "*flash* " *method* is relatively rapid and cheap but is not so reliable and until recently was not officially recognized. A provisional Order, the Milk (Special Designations) Regulations, 1941, however, now permits local authorities to grant licences for the use of the term " pasteurization " for milk which has been heated at a temperature of not less than 162° F. for not less than 15 seconds in an approved apparatus.

Pasteurization is to be considered only as a safeguard against disease transmitted by milk collected with reasonable care ; it is not to be regarded as a means of rendering unclean milk fit for human consumption. It is important to note that the pasteurizing plant must be examined and overhauled regularly in order that it should work efficiently ; irregular results are not infrequently obtained from neglected plants. In view of the recent acceptance of the " flash " method of pasteurization, the regular inspection of plants by competent workers is essential.

Shellfish

Shellfish, such as oysters and mussels, are sometimes cultivated in polluted streams or estuaries and may become contaminated with certain human pathogenic bacteria ; in view of this, care is now taken in the selection of a suitable layering ground. Many cases of enteric fever have followed the consumption of oysters. There is, however, no disease of shellfish communicable to man, contamination is always obtained from an outside source, and it is also possible for contaminated molluscs to become cleansed by placing them in clean water. The bacteriological control of shellfish is consequently an important measure in safeguarding this industry. The usual technique is to take ten oysters from a batch, clean thoroughly, open, remove and cut up the contents and make up to 100 ml. with distilled water; several 1 in 10 dilutions in sterile water are prepared. Material from the various dilutions is added to agar and gelatin plates, MacConkey's broth and freshly boiled litmus-milk. After incubation counts are made and the presence of *Bact. coli* and *Cl. welchii* determined. No official standards are laid down, but it is accepted that when, on the average, more than 100 *Bact. coli* are present per mollusc, the batch is unsuitable for human consumption.

PART III

GENERAL TECHNIQUE

CHAPTER XXXVI

THE MICROSCOPE: STAINING METHODS

THE medical student does not require more than a limited knowledge of bacteriological technique, and for this reason only a brief account of the more important methods is given. Several excellent books have been devoted solely to technique and the reader is advised to consult these if more detailed information is required. The following methods, while perhaps not those described precisely by the originators, have been found by the author to give satisfactory results.

The Microscope

The microscope has been used on many occasions during the earlier studies of the student, but in dealing with bacteria certain points require special attention. Owing to the minute size of bacteria it is practically essential to use a microscope possessing two eye-pieces, 5 times and 10 times, three objectives, $\frac{2}{3}$, $\frac{1}{6}$ and $\frac{1}{12}$ in., a sub-stage condenser, flat and concave mirrors, and some form of mechanical stage. The source of light is also important. Artificial light, such as an opaque, gas-filled, electric bulb, is more suitable than daylight, as it is constant and does not change from day to day. The $\frac{1}{12}$-in. lens is used with cedar-wood oil, which has the same refractive index as glass; this lens is frequently referred to as the oil-immersion lens. The oil is placed on the slide or cover-slip, and the lens is carefully racked down with the coarse adjustment so that the oil is flattened out without the lens coming in contact with the slide. The eye is now applied to the eye-piece and the objective is gradually raised by the coarse adjustment until the smear or sample is brought into view; the final focussing is then carried out with the fine adjustment. When the examination is completed the oil, if moist, should be wiped off the lens with a silk cloth, if dry, xylol is used; alcohol must never be employed for this purpose, as it dissolves the cement in which the lenses are fixed. The oil-immersion lens is used with the plane mirror, an open diaphragm and a raised condenser.

The ⅙ and ⅔-in. lenses are not used to a great extent in the study of bacteria; they are mainly employed in the examination of sections and unstained material, as in the hanging-drop technique for the detection of motility.

With the usual working conditions the range of microscopic resolution is generally about $0{\cdot}25$–$0{\cdot}275\mu$, but it can be increased by the use of ultra-violet light, quartz lenses and photography.

Dark-ground illumination is required for the examination of objects with a low refractive index, such as the spirochætes. By this method direct rays of light are cut out and the specimen is illuminated by oblique rays. This is made possible by the use of a special condenser of the paraboloid or of the concentric reflecting type. An illuminant of high intensity, such as the "pointolite" lamp, is necessary to obtain satisfactory results. If an oil-immersion lens is used a funnel stop is required, as the numerical aperture of the objective must not be greater than 1·0.

In the preparation of the specimen special slides 1·0–1·1 mm. thick and thoroughly clean are used; a thin film is made between slide and cover-slip. A drop of oil is placed on the condenser, the under-surface of the slide and the cover-slip; the condenser is racked up until the drops on the condenser and the under-surface of the slide meet; the objective is then lowered into the oil on the cover-slip and the specimen is focussed. Particulate bodies appear as bright objects against a dark background. When much dark-ground work is carried out it is advisable to have a special microscope and the source of illumination arranged in fixed positions.

Staining Methods

Slides must be well cleaned, free from grease and stored in a stoppered jar containing 50 per cent. methylated spirits. If the slides are greasy it is impossible to make a thin uniform film. The dried smears or films are usually fixed by passing slowly through a bunsen flame. Sections are prepared and treated in a similar manner to those required for histological examination.

Simple Stains

Loeffler's Methylene-blue

1 per cent. aqueous potassium hydrate	1 ml.
Water	99 ml.
Saturated alcoholic methylene-blue	30 ml.

Films are stained for 5 minutes, washed well with water and dried. Sections are stained for at least 10 minutes.

Carbol-fuchsin

Basic fuchsin	1 gm.
Absolute alcohol	10 ml.
5 per cent. aqueous phenol	100 ml.

Carbol-fuchsin when diluted with 19 volumes of water is a useful simple stain, which should be applied for 15–20 seconds only as it tends to overstain.

Differential Stains

Jensen's Modification of Gram's Method

(1) 0·5 per cent. aqueous methyl-violet (6B), 1 minute.

(2) Pour off excess of stain and wash away remains with Lugol's iodine solution, which is allowed to act for 1 minute.

Iodine	1 gm.
Potassium iodide	2 gm.
Distilled water	100 ml.

N.B. Lugol's iodine is three times stronger than Gram's iodine.

(3) Wash off iodine with spirits and continue until no more violet is removed from the film. The time required for this process varies with the nature of the film ; pus takes much longer to decolorize than a bacterial culture.

(4) Wash well with water.

(5) Counter-stain with dilute carbol-fuchsin (1/20) for 20 seconds. Some workers prefer to substitute neutral red (1/1,000) as the counter-stain.

(6) Wash in water and dry between blotting-paper. Gram-positive bacteria take the violet stain and Gram-negative organisms and tissue-cells the counter-stain.

Lillie's Gram Stain for Organisms in Tissues

(1) Tissues are embedded in paraffin in the usual way, sections are cut and brought to water by treatment with xylol and alcohol.

(2) Treat the section with ammonium oxalate crystal-violet solution for 30 seconds.

Crystal-violet	2 gm.
95 per cent. alcohol	20 ml.
1 per cent. aqueous ammonium oxalate .	80 ml.

Dissolve and filter.

(3) Wash in water.

(4) Treat with Lugol's iodine for 30 seconds.

(5) Wash in water.

(6) Decolorize with acetone (10–15 seconds).

(7) Wash in water.

(8) Counter-stain with 0·5 per cent. aqueous safranin for 30 seconds.

(9) Wash in water.

(10) Dehydrate and differentiate with acetone (or alcohol); this removes the excess of safranin and leaves the cell-nuclei deep red and the cytoplasm pinkish, while the organisms are either Gram-positive or -negative; clear with xylol and mount in Canada balsam.

Ziehl-Neelsen Method for Tubercle Bacilli

(1) Cover the slide with carbol-fuchsin, heat until steam rises; the preparation is stained for 5 minutes, heat being applied at intervals. The stain must not be allowed to dry on the slide.

(2) Wash well in water.

(3) Decolorize with 20 per cent. sulphuric acid; the red colour changes to yellowish-brown, but returns on washing in water; decolorization is continued until after washing the smear is a faint pink.

(4) Wash well in water.

(5) Treat with alcohol for 2 minutes.

(6) Wash in water.

(7) Counter-stain with methylene-blue for 30 seconds.

Tubercle bacilli are stained red, while most other bacteria and the tissue-cells take the counter-stain.

Steps (5) and (6) are frequently omitted in the examination of sputum. Treatment with alcohol tends to decolorize some of the non-pathogenic members of the acid-fast group and is particularly useful in the examination of urine and fæces; 3 per cent hydrochloric acid in alcohol (acid-alcohol) is now frequently used as a decolorizing agent.

Neisser's Method (Modified) for Diphtheria Bacilli

Solution A

Methylene-blue	1 gm.
Absolute alcohol	50 ml.
Glacial acetic acid	50 ml.
Distilled water	1,000 ml.

Solution B

Crystal-violet (Höchst)	1 gm.
Absolute alcohol	10 ml.
Distilled water	300 ml.

(1) Mix 2 parts of solution A and 1 part of solution B and stain film for 30–60 seconds.

(2) Wash in water.

(3) Stain with Bismarck-brown (0·2 per cent.) for 30–60 seconds.

(4) Wash in water and dry.

The granules of the bacilli are bluish-black and the protoplasm is light brown in colour.

Moeller's Method for Spores. (1) Carbol-fuchsin, steam for 5 minutes.

(2) Wash in water.

(3) Decolorize by treatment with spirits for a few seconds.

(4) Wash in water.

(5) Counter-stain with methylene-blue for 2 minutes.

(6) Wash in water and dry.

The spores are stained bright red and the bacterial protoplasm blue.

Staining of Capsules

Hiss's Method. (1) Add gentian-violet solution, heat until steam rises and leave for 20–30 seconds.

Saturated alcoholic solution of gentian-violet	1 part.
Distilled water	19 parts.

(2) Wash off the stain with 20 per cent. copper sulphate solution and dry, without washing, between blotting-paper.

The capsules are stained pale bluish-purple and the bacteria deep violet.

Muir's Method. (1) Stain film with carbol-fuchsin for 1 minute, heating gently.

(2) Wash slightly with spirits and then in water.

(3) Treat with the mordant for 30 seconds.

Saturated solution of corrosive sublimate	2 parts.
20 per cent. solution of tannic acid	2 parts.
Saturated solution of potassium alum	5 parts:

(4) Wash well in water.

(5) Treat with spirits for about 20–30 seconds.

(6) Wash well in water.

(7) Counter-stain with methylene-blue for 30–60 seconds.

(8) Wash in water and dry.

The capsules are stained blue and the bacteria red.

Craigie's Method for Staining Flagella. Pipette off the condensation water of a very young agar culture into 2 per cent. formol-saline and allow to fix overnight. Dilute with distilled water and make a thin film on a clean slide; dry and fix by heat at 100° C. for 5 minutes.

Mordant with the following solution for 5–10 minutes at 100° C.

Tannic acid (light and pure)	10 gm.
Tartar emetic (aqueous 5 per cent. solution)	30 ml.
Distilled water	200 ml.

Add a crystal of thymol.

Wash thoroughly in tap water and then in distilled water. Treat with the silver solution, which is prepared in the following manner:

Silver sulphate	1 gm.
Distilled water	250 ml.

To 20 c.c. of this solution add 33 per cent. mono-ethylamine until the precipitate produced just clears. The slide is flooded with this and heated gently until the preparation becomes brown or black.

Wash in distilled water.

Tone with gold chloride solution for 30 minutes in daylight

Gold chloride (1 per cent.)	20 drops
Distilled water	20 ml.

Wash and dry.

Methods for Demonstrating Spirochætes

Fontana's Method. (1) Treat the film three times, 30 seconds each time, with the fixative :

Acetic acid	1 ml.
Formalin	20 ml.
Distilled water	100 ml.

(2) Wash off with absolute alcohol, allowing it to act for 3 minutes ; drain off excess and burn off the remainder until the film is dry.

(3) Add the mordant, heat gently until steam rises and allow to act for 30 seconds.

Mordant

Carbolic acid	1 gm.
Tannic acid	5 gm.
Distilled water	100 ml.

(4) Wash well in water and again dry the slide.

(5) Treat with the silver solution, heating until steam rises, for 30 seconds, when the film becomes brown.

Silver solution. Add 10 per cent. ammonia to 0·25 per cent. solution of silver nitrate drop by drop until the precipitate which forms does not dissolve.

(6) Wash well in water and dry.

The spirochætes are stained brownish-black on a yellowish background.

Levaditi's Method of Staining Spirochætes in Tissues. (1) Fix small pieces of tissue in 10 per cent. formalin for 24 hours.

(2) Wash the tissue for 1 hour in water and then place in 96–98 per cent. alcohol for 24 hours.

(3) Place in 1·5 per cent. silver nitrate solution in a dark bottle and keep in the incubator for 3 days.

(4) Wash in water for 30 minutes.

(5) Place the tissue in the following reducing solution :

Pyrogallic acid	4 gm.
Formalin	5 ml.
Water	100 ml.

and keep in a dark bottle at room-temperature for 48 hours.

(6) Wash well in water, dehydrate the tissue with increasing strengths of alcohol and embed in paraffin. Sections are cut when required and mounted in the usual way.

The spirochætes are stained black and the tissues brown.

Romanowsky Stains

Several modifications of the original Romanowsky stains, particularly Leishman's and Giemsa's, are sometimes used for staining bacteria. These stains contain a mixture of polychrome methylene-blue and water-soluble eosin; these form a precipitate which is washed, dried and dissolved in methyl-alcohol.

Giemsa's stain is used mainly for staining spirochætes and the rickettsiæ; the most satisfactory preparation is that made by Grübler. The smear is fixed by treatment with methyl-alcohol for 3 minutes and the staining process carried out by slow or rapid methods.

In the "rapid method" the stain, diluted with 2 parts of distilled water, is allowed to act for 5 minutes, after which it is washed well with water and dried.

In the "slow method" the stain, diluted with an equal volume of water, is added to a Petri dish, and the slide or cover-slip placed film downwards on the surface of the stain, which is allowed to act overnight. The film is washed in water and dried.

Leishman's stain is usually obtained ready for use and is added to the unfixed smear; the methyl-alcohol acts as a fixative, and is allowed to act for 1–2 minutes, two volumes of distilled water are then added, mixed thoroughly and allowed to act for 5–7 minutes; finally it is washed well in distilled water and dried.

CHAPTER XXXVII

PREPARATION OF CULTURE MEDIA

It has already been mentioned that not only do the growth requirements of different organisms vary, but also that individual species vary according to their age and habitat. It is an accepted fact that many strictly parasitic organisms can be adapted to multiply on the simple media by serial subculture on media of decreasing complexity. The selection of a suitable medium, particularly for the isolation of pathogenic organisms from the tissues, thus constitutes a matter of great importance. It is therefore not surprising to find that a wide range of culture media is available; in fact, it has been estimated that no less than 7,000 different types have been described. It is obviously impossible to be acquainted with even a small proportion of these, but as the majority are essentially modifications and elaborations of certain basic media, it appears most satisfactory to describe the latter in some detail.

In recent years much attention has been directed to the study of bacterial nutrition, and relatively simple *synthetic* media, containing only known chemical compounds, have been introduced. These have, however, proved useful in the study of some pathogenic bacteria, particularly with regard to the production of toxin or enzymes.

For general application culture media are usually prepared from an extract of some animal protein, such as lean muscle. When required different substances, such as peptone, salts, serum, blood, etc., are added to the meat extract. In this way more elaborate media are prepared for special purposes.

After preparation it is necessary to standardize the reaction of the medium to the required *p*H, which is generally about 7·2–7·6. This is carried out by testing colorimetrically 5 or 10 c.c. of the medium against a fixed standard, using a suitable indicator, such as phenol-red. If the medium is too acid or alkaline, decinormal NaOH or HCl respectively is added drop by drop from a burette until the colour agrees with that of the standard. The amount required to correct the reaction of 10 ml. is obtained, and from this figure the volume of normal NaOH or HCl necessary to adjust the bulk of the medium can be estimated.

The cultivation is carried out in glassware of various types, usually in the form of test-tubes, flasks and Petri dishes. These must be thoroughly cleansed before use. New glassware is treated with hydrochloric or nitric acid to remove the free alkali, which is often present. It is then treated with weak caustic soda and well washed in hot water. Old glassware is boiled in a weak soda solution and thoroughly washed in water. When dry the tubes and flasks are stoppered with woollen plugs and all glassware is sterilized in the hot-air oven at 160° C. for 1 hour. After the medium has been added it is usually sterilized either by steaming on three successive days or by treatment in the autoclave at 120° C. for 20–30 minutes.

Meat Infusion Broth. Mince 1 lb. of lean beef, freed from fat, mix with 1,000 ml. of water and leave overnight in a cold place. Then skim the fat off the surface, strain through muslin, squeezing the residue, and after boiling for 15 minutes filter through filter paper; add water to bring the volume back to 1,000 ml.

This constitutes " meat infusion ".

Add 10 gm. of peptone and 5 gm. of sodium chloride.

Adjust the reaction to required *p*H.

Place in tubes or flasks and sterilize in the autoclave.

Infusion Agar. Add 20 gm. of powdered agar to 1,000 ml. of infusion broth; steam for 2 hours; adjust the reaction. If a clear medium is desired, add egg-albumen beaten up in water, steam for 30 minutes, when the egg-albumen is coagulated, bring the volume to 1,000 ml. with water, and filter through paper in the steamer or jacketed funnel. Tube and sterilize in the autoclave.

Gelatin. Add 10–15 per cent. of fine sheet-gelatin (Coignet's Gold Label) to infusion broth, steam for 30 minutes, adjust the reaction, clear with egg-albumen as in the preparation of agar, and filter through paper. Tube and sterilize by the intermittent method or in the autoclave at a relatively low pressure (*e.g.*, 5 lb.). If the temperature is too high, the setting property of gelatin is interfered with.

Peptone-water

Peptone	10 gm.
NaCl	5 gm.
Water	1,000 ml.

Peptone and salt are dissolved by boiling; adjust the reaction; distribute and sterilize in the autoclave.

Peptone-water is used chiefly in testing for the production of indole and as a basis for the fermentation tests.

Hartley's Digest Broth

Lean beef (freed from fat and minced)	1,500 gm.
Water	2,500 ml.
Mix and heat to a temperature of 80° C.	
Add 0·8 per cent. sodium carbonate solution	2,500 ml.
Cool to 45° C.	
Add pancreatic extract	50 ml.
Chloroform	50 ml.

Incubate at 37° C. for 6 hours, stirring frequently.

When digestion is completed, add 40 c.c. of strong hydrochloric acid, steam for 30 minutes and filter.

Adjust the reaction so as to be neutral to phenolphthalein and steam for 20 minutes.

When cool add 0·25 per cent. of chloroform and store in the cold-room.

When required, steam for 20 minutes to remove the chloroform, filter and adjust to the required reaction.

Hartley's broth is a very useful medium, which is widely used for the production of diphtheria toxin.

Loeffler's Serum

Ox, sheep or horse serum	3 parts.
1 per cent. glucose broth	1 part.

Mix, add to sterile test-tubes and inspissate in a sloped position at 75° C. for 6 hours, when the serum coagulates and becomes a yellowish solid.

Sterilize by steaming for 20 minutes on 3 successive days; this is unnecessary if the serum was originally sterile.

Loeffler's serum is mainly used for the cultivation of the diphtheria bacillus.

Serum-agar. Serum, horse, rabbit, etc., should be obtained with sterile precautions. Agar (2 per cent.) is melted and allowed to cool to 45°–50° C., when the sterile serum (5–10 per cent.) is added with a sterile pipette; after mixing the medium is allowed to set either in a Petri dish or as a slope in a test-tube.

Blood-agar. Sterile defibrinated blood is added to 2 per cent. melted agar cooled to 45°-50° C. to give a concentration of 5-10 per cent.; it is allowed to cool in either a Petri dish or a tube in a sloped position.

Chocolate-agar (Boiled blood-agar). Blood-agar medium is heated by immersing in boiling water for 1 minute; it is then allowed to solidify either in a Petri dish or in a test-tube as a slope.

Chocolate-agar is an excellent medium for the cultivation of *H. influenzæ;* heating converts the hæmoglobin into hæmatin and, by breaking down the corpuscles, disperses their contents.

Serum- and Blood-broth. Sterile serum or blood is added to broth by means of a sterile pipette to give a final concentration of 5–10 per cent.

Dorset's Egg Medium. Four fresh eggs are beaten and mixed with 25 ml. of distilled water; the mixture is strained through muslin into test-tubes and then inspissated in a sloped position at 75° C. It is sterilized by steaming on 3 successive days, but should be incubated at 37° C. before use to ensure sterility.

Dorset's medium is used for the cultivation of the tubercle bacillus; 5 per cent. of glycerine may be added.

Potato Medium. Large potatoes are peeled and thoroughly cleaned; a cylinder is removed by means of a borer and is washed in running water to remove excess of starch. The cylinder is cut obliquely into two portions, each of which is placed in a sterile test-tube so that the thick end rests on the bottom. A special type of tube is generally used; this has a constriction a short distance from the bottom, the potato rests on this and some water or broth is placed in the bottom portion to retard drying. The tubes are filled with sterile water and steamed for 30 minutes, the water is subsequently poured off and the tubes are autoclaved for 20 minutes.

Potato, to which 5 per cent. glycerine has been added, is sometimes used for the cultivation of the tubercle bacillus.

Robertson's Meat Medium. Mince 500 gm. of fresh bullock's heart and place in 500 ml. of boiling N/20 NaOH; allow to simmer for 20 minutes. The lactic acid is thus neutralized and the *p*H becomes about 7·5. Drain off the fluid and partially dry the meat on a cloth or filter paper. Then place the meat in sterile test-tubes to fill about 5 cm. of the tube and add broth (*p*H 7·5) until the meat is covered by about 1 cm. Sterilize by autoclaving.

Robertson's medium is extensively used for the cultivation of the anaerobes.

MacConkey's Medium

Peptone (Witte's)	2 gm.
Sodium taurocholate (commercial) .	0·5 gm.
Water (tap)	100 ml.

Steam for 2 hours, filter when hot, allow to stand for 24 hours and filter again.

Dissolve 2 per cent. agar in the solution.

Add a 1 per cent. solution of neutral red to give a reddish-brown colour, and 1 per cent. lactose.

Sterilize by steaming on 3 successive days.

MacConkey's medium is of great value in the isolation of members of the coli-typhoid group, particularly in the examination of fæces. The bile-salt inhibits many other species, while the lactose-fermenters, such as *Bact. coli*, produce pinkish colonies and the non-lactose-fermenters, *e.g.*, *Bact. typhosum*, are greyish.

Dieudonné's Medium. Equal parts of defibrinated ox-blood and normal caustic soda are heated in the steamer.

Three parts of this mixture are added to 7 parts of 3 per cent. agar.

Dieudonné's medium is used for the isolation of the cholera vibrio.

Hiss's Serum-water. One part of serum is mixed with 3 parts of distilled water and a suitable indicator, such as Andrade's, and the sugar solution (1 per cent.) are added.

The mixture is sterilized by steaming for 20 minutes on 3 successive days.

Hiss's serum-water is used in carrying out fermentation tests with certain pathogenic organisms, such as the pneumococcus, which do not grow well in the peptone-water solutions. Fermentation is indicated by the change in the indicator by the production of acid, which may also coagulate the serum.

Andrade's Solution. Add NaOH to a 0·5 per cent. solution of acid fuchsin until the colour just becomes yellow.

This solution is added to the medium to give a concentration of 1 per cent.; the colour is pink when the medium is hot, but disappears on cooling. The indicator is colourless at pH 7·2; when the medium is acid it becomes pinkish.

Litmus-milk. Fresh milk is steamed for 20 minutes and then allowed to stand for 24 hours in order to separate off the cream. The milk is siphoned off and coloured with litmus. It is placed in tubes and sterilized by steaming on successive days.

Litmus-milk is used for biochemical tests. Acid production, due to the fermentation of lactose, turns the medium pink and may also coagulate it; proteolytic activity is indicated by digestion and alkali formation.

McLeod's Chocolate Tellurite Medium. Add to broth, sterilized by filtration, an equal volume of 5 per cent. agar in water, potassium tellurite to make a final concentration of 0·04 per cent., and 7–10 per cent. of freshly drawn defibrinated rabbit's blood; the completed medium is heated at 75° C. for 10–15 minutes and poured into Petri dishes.

This medium is used for the typing of diphtheria bacilli and their isolation from contaminated material, *e.g.*, throat swabs. The potassium tellurite inhibits many organisms, while the diphtheria bacilli produce greyish-black colonies, which can be readily differentiated by an experienced worker from those formed by other organisms.

Tellurite Media. Many other tellurite media are now available for the isolation and typing of diphtheria bacilli. In all the inhibitory substance is potassium tellurite and it has been found that the highest concentration not interfering with the growth of the diphtheria bacillus is 0·04 per cent. The enriching substances in general use are blood and serum and it is important to note that the origin of these is a matter of great importance in type-differentiation. Blood and serum from the rabbit and guinea-pig are most useful; following these in order of merit are sheep, goat, horse and the ox. Horse blood and ox blood, apart from human blood, are least satisfactory but these are considerably improved if the red cells are lysed by freezing and thawing or other means. The usual concentration of the enriching substances is from 5–10 per cent. For routine work on a large scale the use of rabbit or guinea-pig blood is impracticable and consequently sheep blood or serum or lysed horse blood is generally employed.

Semi-solid Medium for the Cultivation of Certain Spirochætes. Mix at 55° C. 8 ml. of sterile saline or Ringer's solution and 1 ml of melted agar; add 20 drops of blood direct from the ear vein of a rabbit; allow to become semi-solid without shaking. Test for sterility by incubating at 37° C. for 24 hours.

This medium is suitable for the cultivation of *Lept. icterohæmorrhagiæ*.

Jensen's Medium for the Cultivation of the Tubercle Bacillus. (1) *Mineral Salt Solution.* Potassium sulphate, 0·4 per cent.; magnesium sulphate, 0·4 per cent.; magnesium citrate, 0·1 per cent.; asparagin, 0·6 per cent.; glycerine, 2 per cent.; distilled water.

Heat the mixture to dissolve the ingredients, then heat at 100° C. for 2 hours.

(2) *Starch Solution.* To every 600 ml. of solution (1) add 30 gm. of potato flour; mix carefully over a water-bath with constant stirring for 15–20 minutes; a paste is produced and is placed in a water-bath at 56° C. for 1 hour.

(3) *Egg-fluid.* Fresh eggs are washed in 5 per cent. soft-soap solution and running water; 20 eggs are required for every 600 c.c. of the medium. The contents of the eggs are mixed by hand for 10 minutes and filtered through sterile gauze. The salt-starch solution is added to the egg-fluid in the proportion of 600 c.c. to 1 litre of egg-fluid.

(4) *Malachite Green.* To each 1,600 ml. of the egg-salt-starch solution add 20 c.c. of a 2 per cent. solution of malachite green. The medium is now distributed before it cools, and the tubes are placed in an inspissator and heated at 85° C. for 30 minutes; after standing overnight at room-temperature they are heated at 75° C. for 30 minutes.

The medium has a rich green colour and is particularly useful for the isolation of tubercle bacilli from contaminated material, such as sputum and fæces. These are subjected to a preliminary treatment with 4 per cent. NaOH (or 6 per cent. H_2SO_4) for about 20 minutes at 37° C.; after which they are centrifuged and the deposit, which may be rendered neutral to litmus, is seeded on the medium.

Wilson and Blair's Medium for the Isolation of Bact. typhosum.

(*a*) Dissolve 6 gm. bismuth ammonio-citrate in 50 ml. boiling distilled water, neutralize with NaOH and cool.

(*b*) Dissolve 20 gm. sodium sulphite crystals in 100 ml. boiling distilled water.

(*c*) Mix (*a*) and (*b*), add 10 gm. anhydrous Na_2HPO_4, boil for 2 minutes and cool.

Solution 1. Add to (*c*) 10 gm. glucose dissolved in 50 ml. water.

Solution 2. Eight per cent. aqueous ferrous sulphate.

Solution 3. One per cent. aqueous solution of brilliant green.

To 100 ml. agar, melted and cooled to 80° C., add 20 c.c. No. 1 solution, 1 ml. No. 2 solution, and 0·5 c.c. No. 3 solution and pour plates. Coliforms tend to be inhibited, while the colonies of *Bact. typhosum* are black.

Russell's Double Sugar Medium

Agar	20 gm.
Peptone	20 gm.
Sodium chloride	5 gm.
Lactose	10 gm.
Glucose	1 gm.
Distilled water	1,000 ml.
Andrade's indicator	10 ml.

Mix ingredients and boil until the agar is completely dissolved.

Restore volume with distilled water.

Adjust reaction to *p*H 7·6.

Tube (10 ml. per tube) and sterilize ; allow agar to set in a slanting position so that there is a deep butt of agar.

Medium is inoculated on the surface and deep into the butt.

Lactose-fermenters produce a general red discoloration usually with bubbles of gas ; the non-lactose-fermenters give a clear surface growth, but there is some red discoloration in the butt. Russell's medium thus provides a simple, rough method of differentiating the main sub-groups of the *Bacterium* genus.

Desoxycholate-Citrate Medium (for the dysentery and Salmonella groups). Hynes' modification of Leifson's medium.

Agar Base. Dissolve 20 gm. lab. lemco in 200 ml. water ; add 50 per cent. NaOH until just alkaline to phenolphthalein, boil and filter ; adjust to *p*H 7·3, restore volume to 200 ml. and add 20 gm. Difco proteose peptone (Evans' peptone is also satisfactory).

Add 90 gm. agar to 3,700 ml. distilled water, steam for 1 hour ; filter and add the lab. lemco-proteose solution.

Add 5 ml. 2 per cent. neutral red and 40 gm. lactose.

Bottle in 100 ml. quantities and sterilize in steam for 1 hour.

Solution A

Sodium citrate (A.R.$Na_3C_6H_5O_7.2H_2O$)	17 gm.
Sodium thiosulphate (A.R.$Na_2S_2O_3.5H_2O$)	17 gm.
Ferric citrate (scales)	2 gm.
Distilled water	100 ml.

Solution is effected by heat or standing at room-temperature for 2 days.

Solution B

Sodium desoxycholate	10 gm.
Distilled water	100 ml.

These solutions need not be sterilized.

For use, add with separate pipettes 5 ml. of solution A and then 5 ml. of solution B to 100 ml. of the melted agar base ; mix well, pour plates and dry.

Selenite Medium (for the Salmonella group).

Sodium acid selenite	4 gm.
Peptone	5 gm.
Lactose	4 gm.
Sodium phosphate	10 gm.
Water	to 1,000 ml.

Adjust to pH 7·1 and sterilize by steaming.

Tetrathionate Broth (for the Salmonella group).

Solution A

Sodium thiosulphate	60 gm.
Water (sterile)	100 ml.

Iodine Solution

Iodine	25 gm.
Pot. iodide	20 gm.
Water	100 ml.

Preparation

Solution A	1·0 ml.
Iodine solution	0·2 ml.
Chalk (sterilized and dry)	0·25 gm.
Broth	9·0 ml.

This medium should be prepared immediately before use and distributed in 5–10 ml. amounts in tubes.

Thioglycollate Medium for Anaerobes (after Brewer).

Infusion broth	1 per cent.
Sodium thioglycollate	0·1 ,, ,,
Agar	0·05 ,, ,,
Glucose	1 ,, ,,

Distribute in tubes (6 in. × $\frac{5}{8}$ in.) in 12 ml. amounts.

Sodium thioglycollate should be freshly prepared by adjusting thioglycollic acid exactly to pH 7·2 with sodium hydroxide.

CHAPTER XXXVIII

SEROLOGICAL TECHNIQUE

Serological reactions can usually be applied in two ways :

(1) In the diagnosis of disease by testing the serum of the patient against known antigens.

(2) To identify an organism (or other antigen) by testing it against known antisera.

The serological reactions most widely used are agglutination, precipitation and complement-fixation, the latter mainly in the form of the Wassermann reaction. The main features and the general application of these reactions have already been considered and we are now only concerned with the technical details. Other tests, such as the opsonic index and the bactericidal test, have also been used, but they have a more limited application.

AGGLUTINATION. Agglutination is probably the most extensively applied of the serological reactions. The test may be carried out by either the macroscopic or microscopic methods ; the latter are, however, less reliable than the former.

Macroscopic Method. The test is carried out in special small tubes which hold about 1·5 ml. and have a pointed end and an open mouth. These are arranged in a small metal rack and are numbered from the left.

Serial dilutions of the serum may be prepared as follows : Add to the first tube 0·9 ml. of saline and to all the others 0·5 ml. ; to the first tube add 0·1 ml. of the serum and mix, then transfer 0·5 ml. of the mixture to tube 2, mix and transfer 0·5 ml. of this mixture to tube 3, and repeat until tube 10 is reached, then after mixing discard 0·5 ml. of the mixture. When several suspensions are being tested against the same serum, it is more satisfactory to prepare larger quantities of the dilutions separately and to add 0·5 c.c. of each dilution to the corresponding tube of the test. Next, using a fresh pipette, add to all tubes 0·5 c.c. of the bacterial suspension, mix by shaking and place in a water-bath at 50° C. so that only half the column of fluid is immersed (Table XXXII.). Examine after varying intervals of time, usually 1, 4 and 24 hours, preferably by holding in front of a shaded lamp set against

TABLE XXXII

Scheme of Agglutination Technique

TUBE.

Contents	1	2	3	4	5	6	7	8	9	10	11
Saline (ml.)	0·9	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5
Serum (ml.)	0·1 →	0·5 →	0·5 →	0·5 →	0·5 →	0·5 →	0·5 →	0·5 →	0·5 →	0·5 → discard 0·5 ml.	—
Bacterial suspension (ml.)	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5
Total volume (ml.)	1	1	1	1	1	1	1	1	1	1	1
Serum dilution (final)	1/20	1/40	1/80	1/160	1/320	1/640	1/1,280	1/2,560	1/5,120	1/10,240	—

a dark background; the differences in the reactions given by O and H antigens have already been noted.

In this country bacterial suspensions can be obtained from the Standards Department, Central Public Health Laboratory, Colindale; these are carefully standardized so that readings obtained in different laboratories are strictly comparable. If standard suspensions are not available, a simple method of preparation is to heat a saline suspension of an agar slope culture of the organism at 56° C. for 30 minutes; with motile organisms the H factor is usually the predominant antigen.

Reading. Tube 11 is the control tube, in which the bacterial suspension is tested for spontaneous agglutination. This is examined first; clumping, *i.e.*, spontaneous agglutination, should not occur, as in such an event the test would be unsatisfactory. The other tubes are then examined and the following points noted: The presence and type of agglutination and its time of appearance—H agglutination is rapid and floccular; the O type is slow and granular. All tubes should be examined, as the prozone phenomenon may be present. In such cases agglutination may not be present in tubes 1 and 2, *i.e.*, those containing the greatest concentration of serum, but may be well marked in other tubes. The titre is taken to be the highest dilution at which definite agglutination takes place.

The Microscopic or Slide Method. A drop of the bacterial suspension is mixed with a drop of the serum (undiluted or diluted 1/10); after standing a few minutes the mixture is examined with the hand lens or the low power of the microscope. In a positive reaction clumping is well marked.

PRECIPITATION. In this test the antigen is diluted and the antiserum kept constant. The mixtures are prepared as for the agglutination reaction, but the tubes are incubated at 37° C. The test is frequently used for the identification of blood stains. It is also applied in bacteriology, *e.g.*, in typing pneumococci in cases of pneumonia; the urine of these cases frequently contains the specific carbohydrate, and using this as the antigen it may be tested against the three type sera.

A modified precipitation or flocculation test is used extensively in the diagnosis of syphilis; many forms have been described, *e.g.*, Sachs-Georgi, Meinicke, Sigma, Kahn, etc.; of these the Kahn test is probably the most satisfactory and reliable.

Kahn Test. If the complete details are required, reference should be made to the original monograph. The following reagents are required: (1) antigen, (2) patient's serum, and (3) saline.

(1) The "antigen" is an alcoholic extract of the ether-insoluble lipoids of beef heart. This is prepared by treating repeatedly the dried muscle with ether; the dried muscle is extracted with alcohol, the extract is then cholesterolized (0·6 per cent.) and filtered. Before use it must be standardized. The standard antigen is prepared by Messrs. Parke, Davis & Co. When required it is mixed with saline according to the titre, the usual proportion being 1 ml. of antigen to 1·2 ml. of saline, in special tubes by pouring the saline into the antigen; the pouring operation is repeated twelve times. The diluted antigen is allowed to stand for ten minutes, but must be used within 30 minutes of preparation.

(2) The patient's serum is heated at 56° C. for 30 minutes.

The test is carried out in special small tubes, 7·5 × 1·0 cm., according to the scheme given in Table XXXIII. Owing to the minute quantities involved special graduated 1·0 c.c. and 0·1 c.c. pipettes are required.

TABLE XXXIII

The Kahn Test

	Tube 1	Tube 2	Tube 3	Tube 4
Antigen (ml.)	0·05	0·025	0·0125	0·05
Serum (ml.)	0·15	0·15	0·15	—
	Shake vigorously, at about 280 oscillations per minute, for 3 minutes and then add the saline.			
Saline (ml.)	1·0	0·5	0·5	1·0

After the addition of the saline, which is added to simplify the detection of flocculation, the tubes are lightly shaken and read. The degree of flocculation is indicated by a scale varying from ++++, indicating the formation of visible particles, to —, denoting uniform opalescence, which should be given by control tube 4. The test is not quantitative, each of the three tubes is equally significant, and for the final interpretation of the result an average of the three tubes is taken. A scale indicating the value of the various readings has been adopted by Kahn; a positive result is one in which the average reading is ++ or over.

The Kahn test is sensitive and relatively simple; however, while results of ++++, +++ and − values are easy to read, those of ++ and + values may require considerable experience to detect; a hand lens and a special viewing box should be used. The technique required for the examination of cerebrospinal fluid is much more complicated than that required for serum.

COMPLEMENT FIXATION. Complement fixation is used mainly in the diagnosis of syphilis, as the Wassermann test. It is also sometimes applied in the diagnosis of gonorrhœa, tuberculosis and certain virus diseases; in these cases the technique is similar to that of the Wassermann test, the chief difference being in the nature of the antigen.

The Wassermann Reaction. The principles of the reaction have already been discussed. Sensitized sheep cells are used to indicate the fixation of complement by the antigen and the patient's serum. If complement is fixed, *i.e.*, the test is positive, the cells are not lysed; if the test is negative the complement is free and the blood-cells are lysed.

The reagents required for this test are (1) antigen, (2) patient's serum, (3) complement, (4) sheep corpuscles, (5) antisheep hæmolysin, and (6) saline.

(1) The *antigen* is a cholesterolized alcoholic extract of human heart muscle. To prepare, macerate 10 gm. of lean heart muscle in 100 ml. of absolute alcohol; filter through paper and store in the dark. Prepare a 1 per cent. solution of cholesterol in alcohol. These are kept in separate bottles and are mixed on the day of the test in the following manner: three parts of heart extract and 2 parts of cholesterol solution are mixed in a large test-tube; a 1/15 dilution of the mixture is made by the rapid addition of 14 parts of saline.

(2) The *patient's serum* is carefully labelled and heated at 58° C. for 20 minutes on the morning of the test to destroy the complement; it should be free from hæmoglobin.

(3) *Complement* is obtained by bleeding guinea-pigs the day before the test; the blood is allowed to clot and the serum collected. Complement is unstable and, unless kept in a frozen or dried state, can only be used on the day of the test.

(4) *Sheep corpuscles.* Sheep are bled from the jugular vein into a vessel containing beads; the blood is defibrinated by

shaking vigorously for 5–10 minutes, strained through muslin, washed in saline and centrifuged three to five times, finally a 5 or 10 per cent. suspension is made from the deposit. The concentrated cells keep about 3 days at 0° C.

(5) *Antisheep hæmolysin* is prepared by injecting rabbits with sheep corpuscles: the injections should be continued until a trial test indicates that a satisfactory serum, *i.e.*, one with an M.H.D. of 1/5,000 or more, has been obtained. The animal is then bled, the serum collected and stored at 0° C. The hæmolysin is very stable and keeps for years with only a slight reduction in titre.

Standardization of Reagents. Before the actual test can be performed certain preliminary titrations must be carried out. The antigen and hæmolysin exhibit little variation from day to day and, in routine work, it is only necessary to make a preliminary titration of each batch of complement.

Titration of Complement. This is done to determine the M.H.D. (minimal hæmolytic dose) of the complement, *i.e.*, the smallest amount which completely lyses the unit volume of sensitized cells in 1 hour at 37° C. Serial dilutions of complement are tested against constant amounts of sensitized cells. In all preliminary titrations and the actual test a unit volume of each reagent (usually 0·5 ml.) is used.

A 1/5 dilution of the fresh guinea-pig serum is prepared by adding 0·5 ml. of the serum to 2 ml. of saline. Set up a row of tubes and add, in succession from the first tube, the following amounts of saline in ml.: 0·75, 1, 1·25, 1·50, 1·75, 2·25, 2·75, 3·25. Using a fresh pipette, add to each tube 0·25 ml. of the 1/5 dilution of guinea-pig serum. This gives the following series of dilutions: 1/20, 1/25, 1/30, 1/35, 1/40, 1/50, 1/60, 1/70.

Set up a rack containing eight special Wassermann tubes and number from the left. With a fresh pipette add 0·5 ml. of the 1/70 dilution of complement to tube 8; then mix the 1/60 dilution and add 0·5 ml. to tube 7; continue to the 1/20 dilution.

Using a fresh pipette for each reagent, add to each tube 1 ml. of saline, 0·5 ml. of hæmolysin (3 M.H.D.) and 0·5 ml. of a 5 per cent. suspension of red blood corpuscles. Incubate at 37° C. and read after 1 hour; the dilution of complement producing complete hæmolysis is the M.H.D. In the final test 3 M.H.D. of complement are required; if 1 M.H.D. is a dilution of 1/45, 3 M.H.D. represents a dilution of 1/15.

Titration of Antigen. Each fresh batch of antigen is tested to ensure that it is not too anticomplementary. In carrying out this test two complement titrations are necessary, the standard method and another in which 0·5 ml. of saline is replaced by 0·5 ml. of the diluted antigen.

A satisfactory antigen is one with slight anticomplementary activity but with a high degree of sensitivity to syphilitic sera.

Titration of Hæmolysin. It is necessary to test the hæmolysin at regular intervals. This is carried out in a similar manner to the titration of complement. The main difference is that the dose of complement is kept constant at 3 M.H.D. and various dilutions of hæmolysin are prepared. The dilution giving complete hæmolysis after 1 hour at 37° C. is the M.H.D.

TABLE XXXIV

The Wassermann Reaction

	Tube 1	Tube 2	Tube 3	Tube 4
Serum (inactivated) (ml.)	0·5 (1/2)	0·5 (1/2)	0·5 (1/8)	0·5 (1/32)
Complement (3 M.H.D.) (ml.) . . .	0·5	0·5	0·5	0·5
Antigen (1/15) (ml.) .	—	0·5	0·5	0·5
Saline (ml.) . . .	0·5	—	—	—
	Incubate at 37° C. for 1 hour.			
Hæmolysin (3 M.H.D.) (ml.) . . .	0·5	0·5	0·5	0·5
Corpuscles (5 per cent.) (ml.) . . .	0·5	0·5	0·5	0·5
	Incubate at 37° C. for 1 hour and then leave overnight at 0°–4° C.			

The Wassermann Test. Set up four tubes in a rack and label carefully. The first is the serum control (dilution 1/2) and does not contain antigen, saline being the substitute ; the others are for the serum dilutions 1/2, 1/8 and 1/32 and require antigen. Complement is added to all, and the tubes incubated at 37° C. for 1 hour. Unit volumes of hæmolysin and corpuscles are then added and the tubes again incubated at 37° C. for 1 hour (see Table XXXIV.).

Results. Readings are easier to make if the tubes have been left overnight in the refrigerator. The absence of lysis indicates a positive, complete lysis a negative, and incomplete lysis a doubtful result. A quantitative reading is obtained by readings of the different serum dilutions. The control tube should show complete lysis, otherwise the serum is anticomplementary and therefore unsuitable for examination. When only one serum is being tested an antigen control must also be made. This is usually omitted in routine work as the antigen is known to be satisfactory, and, when many tests are done, some are negative and these serve as controls. Only a slight modification is required for the examination of cerebrospinal fluid, this is in the preparation of the dilutions of the fluid.

Clinicians usually prefer a quantitative system of recording the result, such as + 1/32, + 1/4, or doubtful 1/2. The League of Nations Committee has, however, recommended that the results should be recorded as + (positive), ± (doubtful), and — (negative).

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